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POSGRADO EN CIENCIAS BIOLÓGICAS

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FILOGENIA MOLECULAR DEL HONGO FITOPATÓGENO *RHIZOCTONIA SOLANI* BASADA EN LAS SECUENCIAS DEL ADN RIBOSOMAL NUCLEAR (ITS Y LSU) Y DEL GEN BETA-TUBULINA

T E S I S

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DOCTORA EN CIENCIAS (BIOLOGÍA)

PRESENTA

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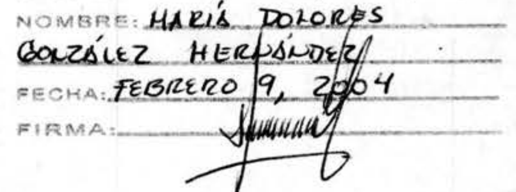
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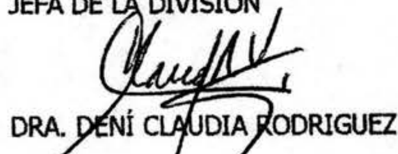

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RESUMEN

El complejo de especies del hongo fitopatógeno *Rhizoctonia solani* (teleomorfo = *Thanatephorus*) consiste al menos de 14 grupos anastomósicos. Para evaluar si el complejo y cada grupo anastomósico son monofiléticos se diseñaron análisis filogenéticos basados en parsimonia. Se secuenció la región intergénica del ADN ribosomal para 43 representantes de *R. solani* y 16 de *Rhizoctonia* binucleados (teleomorfo = *Ceratobasidium*). A éstas se agregaron 63 secuencias de GenBank para un primer análisis. Posteriormente, se secuenciaron el 28S y el gen beta-tubulina en 26 representantes de *R. solani* y 17 de *Rhizoctonia* binucleados para un segundo análisis. Con los datos combinados se realizaron análisis de sensibilidad de las topologías excluyendo las posiciones con indels e incluyéndolas codificadas como caracteres ambiguos. Para explorar si son fuente de información filogenética, se codificaron las indels de más de dos nucleótidos como: 1) multiestados para cada secuencia, 2) multiestados para cada longitud, y 3) como diferentes caracteres para cada secuencia distinta. El efecto de los distintos códigos se midió en la resolución, topología, número de árboles, congruencia de caracteres entre juegos de datos y mediante índices de robustez. Los cladogramas más resueltos y con mejor apoyo se obtuvieron cuando las indels se incluyeron en los análisis. Las indels recodificadas como diferentes caracteres rescataron mejor la información histórica. Los resultados indicaron que el complejo *R. solani* tal como se clasifica actualmente no es monofilético. Siete clados con los representantes de los grupos anastomósicos 1, 4, 6, 8, 10, BI y el subgrupo 2-2 de *R. solani* así como cinco clados de *Ceratobasidium* se recuperan en todos los análisis con elevados índices de apoyo lo que indica que son grupos naturales. Por el momento se propone que sólo los clados con representantes de los grupos anastomosicos 1, 4, 6 y 8 sean reconocidos al nivel de especie. Los resultados sugieren que las indels en regiones no codificadoras contienen información filogenética.

ABSTRACT

The species complex of the plant pathogenic fungi *Rhizoctonia solani* (teleomorph = *Thanatephorus*) consists at least of 14 anastomosis groups. Phylogenetic analyses using parsimony were designed to evaluate if the complex and each anastomosis group are monophyletic. The non-transcribed intergenic region of the ribosomal DNA was sequenced for 43 isolates of *R. solani* and 16 binucleate *Rhizoctonia* (teleomorph=*Ceratobasidium*). To these, 63 sequences from GenBank were added for a first analysis. Next, the 28S and the beta-tubulin gene were sequenced in 26 isolates of *R. solani* and 17 of binucleate *Rhizoctonia* for a second analysis. Sensitivity analyses on topology were performed with the combined data set excluding all positions with indels and including them as ambiguous characters. To explore if they are a source of phylogenetic information, indels of more than one nucleotide were coded as: 1) multistates for different sequence, 2) multistates for different length and 3) as different characters for each distinct sequence. The effect of different coding was measured on resolution, topology, tree number, character congruence among data sets and support indexes. The clades with best resolution and with better support were obtained when indels were included in the analyses. The indels recoded as different characters recovered better the historical information. Results indicated that *R. solani*, as it is classified today, is not monophyletic. Seven clades with isolates of the anastomosis groups 1, 4, 8, 10, 6 and BI and subgroup 2-2 of *R. solani* and five clades of *Ceratobasidium* are recovered in all analyses with high support indexes indicating they are natural groups. At the moment, we suggest that only clades with representatives of anastomosis groups 1, 4, 6 and 8 be recognized as species. Results suggest that indels in noncoding regions contain phylogenetic information.

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INTRODUCCION

La clasificación de los organismos es una disciplina biológica que se ha desarrollado bajo una variedad de fundamentos filosóficos. Aunque estos fundamentos tienen como tema central el comprender el orden de la diversidad biológica (Bock, 1974), a través de la historia no ha existido un criterio unificado sobre los procedimientos de análisis para ordenar esta diversidad. A partir de la teoría de la evolución propuesta por Darwin, los esfuerzos de muchos biólogos se centraron, por un lado, en descubrir los mecanismos biológicos que producían los cambios en los organismos y por el otro, en encontrar una explicación del orden entre ellos.

Hasta la primera mitad del siglo veinte era común que un especialista dedicara años a estudiar un grupo particular y sobre la base de su experiencia acumulada asignaba jerarquía taxonómica a sus organismos. Ésto, aunque contribuyó a catalogar la diversidad del mundo natural, originó dificultades debido a que se carecía de métodos estándares tanto conceptuales como operativos para la clasificación (Avise, 1994), lo que trajo como consecuencia: 1) la centralización de "sistématas autoridades" dentro de un número pequeño de investigadores para cada grupo taxonómico; 2) la falta de procedimientos formales para corroborar o refutar una clasificación propuesta; 3) la ausencia de medidas uniformes para comparar significativamente la clasificación de grupos taxonómicos diferentes; y 4) la falta de una orientación filosófica clara sobre cuáles aspectos de la evolución se reflejaban en una clasificación particular (Avise, 1994). Sin embargo, a partir de los años 1960s, se generó un intenso debate sobre los aspectos conceptuales y metodológicos, desde el punto de vista filosófico, que están involucrados en una clasificación. Esto originó que se distinguieran claramente tres escuelas de sistemática con distintos enfoques: la evolutiva, la fenética y la cladista (Bock, 1974). Actualmente, todos los estudios taxonómicos que se realizan, involucran conceptos y teorías de alguna de estas tres escuelas. La presente investigación se desarrolló bajo un enfoque metodológico cladista.

Enfoque cladista de la sistemática

La sistemática cladista incorpora los principios metodológicos del racionalismo crítico popperiano (Popper, 1991), mediante la refutación de hipótesis filogenéticas que se usan para establecer una clasificación. Esa hipótesis se pone a prueba mediante el examen de la distribución congruente de las sinapomorfias (rasgos compartidos-derivados). Consecuentemente, la hipótesis que sobrevive en la prueba no es la mejor verificada, sino es la menos refutada por la evidencia conflictiva de similitudes no homólogas. Esto significa que, entre varias hipótesis, se prefiere la que requiere el menor número de proposiciones de homoplasia (rasgos similares que no reflejan historia evolutiva común, Hull, 1983; Kitts, 1977; Ruse, 1979; Wiley, 1975).

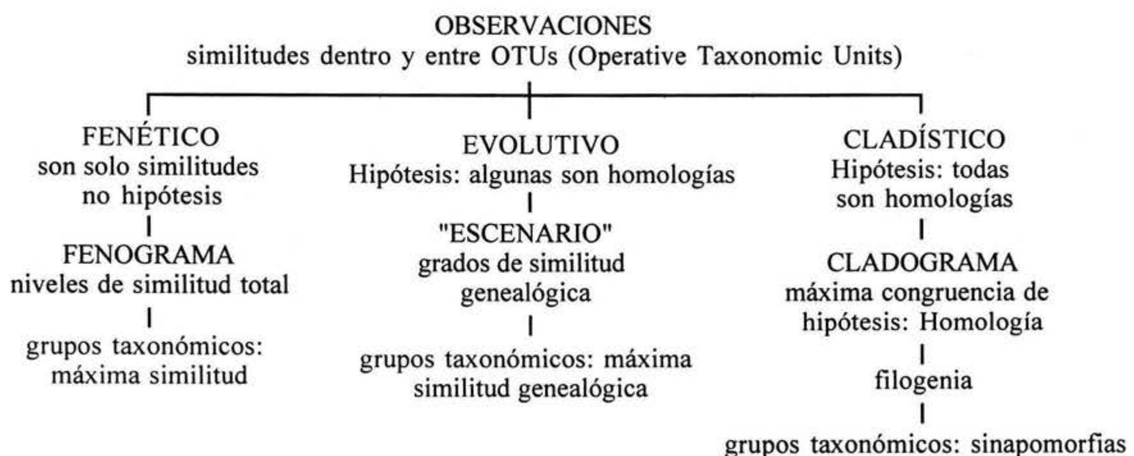
El enfoque cladista está basado en una ontología evolutiva y es compatible con el concepto de los taxa como individuos. Ontológicamente, las especies o los grupos monofiléticos son unidades discretas que están restringidas espacio-temporalmente. Epistemológicamente estos grupos pueden descubrirse mediante el patrón congruente de similitudes heredables a todos los componentes del grupo. Este subjuego particular de similitudes que revelan la conexión histórica entre los componentes del grupo son, precisamente, las sinapomorfias. La existencia, continuidad y cohesividad de los componentes del grupo están en función de procesos biológicos como el aislamiento

reproductivo y no de la condición de que los componentes incluidos posean ciertos atributos que definen los grupos taxonómicos como propone la escuela fenética (Hull, 1978; Patterson, 1978; Platnick, 1977; Rieppel, 1986; De Luna, 1995).

El enfoque cladista de la sistemática además propone que es necesario hacer inferencias durante la elaboración de una clasificación. Las homologías, los grupos monofiléticos y las relaciones filogenéticas están fuera del dominio empírico, pero de acuerdo con la teoría evolutiva son reales y entonces se pueden inferir a partir del análisis de las similitudes heredables y pueden usarse como base para la clasificación (De Luna, 1995). La congruencia entre las inferencias independientes de las homologías así como las inferencias relativas a las transformaciones de caracteres sinapomórficos son la base para las hipótesis filogenéticas (Humphries y Funk, 1984). En este caso, tanto las sinapomorfias como las hipótesis de filogenia, pueden inferirse y examinarse mediante el principio de parsimonia (Beatty y Fink, 1979; Farris, 1983; Kluge, 1984) logrando suficiente rigor metodológico como para usar estas hipótesis en la construcción de clasificaciones. El enfoque cladista se cataloga como histórico, pues la clasificación se deriva de las filogenias inferidas (Cracraft, 1983).

Método cladista para la elaboración de una clasificación.

La clasificación biológica nos ayuda a catalogar información de los organismos a través de un sistema ordenado de nombres (Lundberg y MacDade, 1990). Sin embargo, la forma de clasificar así como el tipo de información que se cataloga difiere entre las escuelas fenética, evolutiva y cladista. Algunos sostienen que las clasificaciones sólo deben contener la información de los caracteres que son diagnósticos para un grupo particular, mientras que otros aseguran que las clasificaciones son un medio para expresar hipótesis de interrelaciones. La metodología para elaborar una clasificación también es distinta en cada una de estas escuelas.



Enfoques para la clasificación en las tres escuelas de Sistemática

La escuela cladista construye hipótesis de interrelaciones que están corroboradas por caracteres independientes. Se considera que cada especie está sujeta a eventos evolutivos únicos que cambian rasgos ancestrales (plesiomórficos) a rasgos derivados (apomórficos). Los rasgos derivados permiten reconocer grupos monofiléticos. Esto se debe a que

constituyen marcadores de la historia evolutiva común de un linaje en el cual ese rasgo evolucionó, y por ende en todos sus descendientes. En una filogenia que involucra a más de dos grupos, cada rasgo que un organismo comparte se atribuye a una de las tres clases de similitudes: sinapomorfias, simplesiomorfias (similitudes primitivas compartidas) y homoplasia.

Una vez que el grupo de estudio ha sido seleccionado, se examinan comparativamente varias muestras de ese taxón en los rasgos que varían entre ellos. En sistemática, un carácter es cualquier aspecto del fenotipo o genotipo que varía dentro del grupo de estudio. Un estado del caracter es la expresión de un caracter encontrado en la unidad taxonómica bajo estudio. La unidad de un taxón puede ser monomórfico o polimórfico para un estado de caracter dado.

Cuando la información de los caracteres se ha ensamblado, y se han hecho todas las decisiones de los estados de los caracteres durante el proceso del análisis de los caracteres se forma una matriz "caracter por taxon". Se generan hipótesis filogenéticas al reconocer los grupos monofiléticos basados en el principio de Hennig (1966) (los cambios evolutivos heredables se transmiten de los ancestros a los descendientes). El formato que se usa para presentar una hipótesis filogenética, por lo general, es un diagrama en forma de árbol. Si hay poco o ningún conflicto en las relaciones genealógicas que sugieren los datos, las relaciones filogenéticas se infieren inmediatamente del patrón de sinapomorfias (Lundberg y McDade, 1990). Los taxa más cercanamente relacionados compartirán el mayor número de caracteres derivados. Sus grupos hermanos compartirán solo algunos de esos caracteres, y así sucesivamente hasta que se resuelven las relaciones de todos los taxa. Cuando hay conflictos en los caracteres o incongruencia, es indicación de que por lo menos algunos de los estados de caracteres no son homólogos debido a paralelismos, convergencias o a errores durante el análisis de los caracteres (Lundberg y McDade, 1990; Wiley *et al.*, 1991).

El problema de la clasificación en hongos anamorfos.

Uno de los problemas más serios en la clasificación de hongos anamorfos es la definición de las especies. Aunque este es uno de los problemas más grandes a los que se enfrenta la biología en general y la sistemática en particular, el desarrollo de una teoría filogenética ha estimulado a reconsiderar nuevamente la naturaleza de las especies y la manera como deben dirigirse los estudios empíricos a fin de descubrirlas (Davis, 1995).

El reconocimiento de las especies como individuos ontológicos permitió separar dos ideas en la conceptualización de las especies; primera, qué son las especies y segunda cómo se reconocen. Como consecuencia, surgieron los conceptos históricos de especie. Estos conceptos consideran a las especies retrospectivamente como producto de la evolución y la definen como organismos conectados históricamente. Los conceptos históricos surgieron como resultado del desarrollo de una teoría rigurosa de la reconstrucción filogenética y de la incorporación de caracteres moleculares (Davis, 1995). La reconsideración del problema de la especie, con atención a la teoría filogenética y a la variación molecular, ha propiciado la introducción de conceptos nuevos desarrollados específicamente dentro del dominio de la sistemática filogenética.

Al igual que para otros organismos, para los hongos no existe un concepto universal de especie. Aunque se han aplicado diversos conceptos dependiendo de la posición ontológica que se tenga, éstos tienen limitaciones. Por ejemplo, si se intenta aplicar el concepto ecológico de especie, surgen problemas empíricos. Esto se debe a que en muchos hongos no hay una relación evidente del nicho ecológico (Van Valen, 1976; Brasier, 1987;

Crisci, 1994). También, al tratar de aplicar el concepto esencialista (Ruse, 1988; Sober, 1993; Mishler y De Luna, 1997) para reconocer a las especies se observa que muchos hongos tienen una morfología muy simple. Como consecuencia, los patrones de variación morfológicos aportan poca información para el reconocimiento de las especies (Vilgalys, 1991). Aunque muchos micólogos declaran que aplican el concepto biológico de especie realmente el método es esencialista pues enfatizan las estructuras reproductoras para reconocer a las especies (por ejemplo Fries, 1985; Boidin, 1986).

El problema de la definición de especie en hongos anamorfos es todavía más grave. Una forma de resumir mas de 100 años del pensamiento filosófico concerniente a los hongos anamorfos es con las palabras de Weresub y Pirozynski (1979, citado en Reynolds, 1987) "...la forma apropiada de tratar formas asexuales es como incompletas, potencialmente 'perfectas' restringiendo el término y concepto de especie y nomenclatura botánica a organismos sexuales". Esta idea ha sido apoyada históricamente por el uso de términos como "hongos asexuales" y "hongos imperfectos". Weresub y Pirozynski reflejan el sentimiento de muchos otros investigadores que enfatizan las estructuras reproductoras como el primer caracter para reconocer las especies de los hongos bajo un concepto esencialista (morfológico en este caso).

En algunos hongos anamorfos, las estructuras sexuales pueden inducirse artificialmente en cultivo. De esta forma se puede establecer la relación entre un estado sexual (teleomorfo) y un anamorfo (Muller, 1987; Adams, 1988; Tsuda y Ueyama, 1987). Pero para los hongos que no producen esporas en cultivo, la conexión anamorfo-teleomorfo es elusiva, y a menudo solo se determina intuitivamente. De esta forma, cualquier conclusión acerca del reconocimiento de la especie como unidad morfológica es solo tentativa.

En el contexto del concepto biológico de especie, es importante el establecimiento de una prueba sólida de las relaciones teleomorfo-anamorfo para el reconocimiento válido de la especie. Sin embargo, existen diversos problemas empíricos para demostrar una conexión teleomorfo-anamorfo. Algunas veces se dice que los teleomorfos y anamorfos pertenecen al mismo holomorfo (estado perfecto) basados en la observación de cohabitación o algunas suposiciones de "homología" morfológica, pero sin evidencia directa de una conexión biológica entre las dos fases (Reynolds, 1987). Si tal conexión existiera, proveería el marco para la reevaluación del reconocimiento de las especies. Sin embargo, estos casos son raros comparados con los cientos que nunca desarrollan el estado teleomorfo y entonces no pueden ser identificados.

Los argumentos anteriores llevan a la conclusión de que los estudios de los hongos anamorfos parecen estar restringidos por problemas empíricos y los derivados de la aplicación del concepto biológico de especie. Una solución posible es mejorar los métodos para inducir el estado sexual en todos los hongos anamorfos. Pero también es posible que otro concepto de especie, aun careciendo de la fase sexual, pudiera ayudar en desarrollar un mejor entendimiento de los patrones de variación. En esta dirección, la consideración de un concepto histórico de especie con enfoque filogenético es prometedora para los hongos anamorfos.

Nuevos enfoques para la clasificación de hongos anamorfos.

Muchos hongos de los que se desconoce su fase sexual son importantes en la agricultura, medicina e industria (Bold *et al.*, 1980; Moore-Landecker, 1996; Pointing y Hyde, 2001). A pesar de su importancia, su taxonomía es incierta debido a que los

caracteres sexuales, tradicionalmente usados en estudios taxonómicos en los hongos, no están presentes. Por este motivo, es necesario reconsiderar dos aspectos para la clasificación de estos hongos: 1) la posibilidad de explorar caracteres adicionales a los morfológicos en la fase asexual, y 2) el uso de métodos más robustos de análisis que permitan establecer su taxonomía, basada en las relaciones filogenéticas de este grupo de hongos.

En los últimos años, el desarrollo de nuevos enfoques de la sistemática ha seguido dos caminos paralelos. Uno de ellos ha introducido los caracteres moleculares, y el otro, ha introducido nuevos tipos de análisis como son los cladísticos (González, 1997). Sin embargo, recientemente se observa un incremento en el uso los análisis de máxima verosimilitud y bayesianos aplicados a los caracteres moleculares.

El interés de los micólogos también ha aumentado considerablemente tanto para usar los caracteres moleculares como los nuevos tipos de análisis. Como resultado se han podido comparar, desde una perspectiva filogenética, muchos grupos de hongos (Bruns *et al.*, 1991, 1992; Vilgalys *et al.*, 1991; Bowman *et al.*, 1992; Swann y Taylor, 1993; 1995; Taylor, 1995a, Hibbett, 1997, Hibbett y Thorn, 2001; Moncalvo *et al.*, 2000, 2002; Binder y Hibbett, 2002). Aunque algunas relaciones que se han deducido cladísticamente con caracteres moleculares han sorprendido a los micólogos (Swann y Taylor, 1995), a menudo los estudios filogenéticos con ácidos nucléicos han servido para confirmar lo que ya se había inferido con la morfología (Taylor, 1995a). Además, donde hay conflictos derivados de caracteres morfológicos, los caracteres moleculares han contribuido a esclarecer las clasificaciones (Bruns *et al.*, 1991).

Los primeros estudios comparativos con un enfoque molecular en hongos anamorfos datan de mediados de los 80's (Walker y Doolittle, 1983). A partir de entonces, se observó que las secuencias del ADN representaban un gran potencial para conectar los anamorfos y los teleomorfos. Gaudet y colaboradores (1989) integraron varias especies del anamorfo *Fusarium* y sus teleomorfos *Gibberella*, *Calonectria* y *Nectria* usando secuencias de la subunidad corta del ADN ribosomal (Taylor, 1995b). La conexión de anamorfos con teleomorfos trajo como consecuencia que se cuestionara, inclusive, la existencia del grupo de los Deuteromycetes: "if all fungi can be compared through their nucleic acids and placed on a single phylogenetic tree, do we need to maintain the Deuteromycota?" (Bruns *et al.*, 1991). A partir de entonces, el interés creció rápidamente para realizar estudios filogenéticos con ácidos nucléicos que combinaran deuteromicotes (particularmente los económicamente importantes) con varios teleomorfos (Reynolds y Taylor, 1993).

La tarea del micólogo interesado en hongos anamorfos es hacer un esfuerzo por conectarlos con los teleomorfos en un sólo sistema de clasificación. Sin embargo, es importante señalar que esta clasificación no está garantizada simplemente por el uso de datos moleculares sino por un manejo adecuado de los datos en un nuevo enfoque para su análisis. A este respecto, una clasificación basada en las relaciones filogenéticas de este grupo de hongos sería la más apropiada.

Para que un sistema de clasificación se considere filogenético, sólo deben reconocerse grupos monofiléticos a partir de caracteres derivados que reconstruyan las relaciones de ancestría común. Los taxa que no son monofiléticos deben rechazarse y reagruparse para reflejar jerarquía filogenética. Obviamente a corto plazo, habrá resistencia a la implementación de sistemas filogenéticos por muchos micólogos. Pero a largo plazo, el valor de estos sistemas va a resultar en una clasificación más robusta y estable que refleje más cercanamente las relaciones naturales (Vilgalys y Hibbett, 1993).

Una de las grandes fortalezas del sistema filogenético es que los métodos y resultados son transparentes, lo que significa que las decisiones buenas o malas se pueden examinar. El sistema filogenético no depende de algún conocimiento especial de los organismos que solo el "experto" pueda entender. Un crítico no puede reclamar que una idea de la historia filogenética de un grupo esta equivocada solo porque él ha estudiado los grupos más tiempo. Desde luego que hay desacuerdos válidos, y se pueden hacer cambios y mejoras. Pero estos desacuerdos están basados en un análisis de los datos y no en opiniones personales.

Objetivos

En esta tesis se usaron métodos de parsimonia con caracteres moleculares para evaluar si los diferentes grupos anastomósicos del complejo de especies del hongo fitopatógeno *Rhizoctonia solani* (teleomorfo = *Thanatephorus*) representan linajes evolutivos independientes, y para proponer una hipótesis de relaciones filogenéticas dentro de este hongo.

Además se evaluó si las inserciones-deleciones (indels) originadas durante el alineamiento de las secuencias, son informativas filogenéticamente. Para ello, se realizó un primer análisis con los grupos anastomósicos del AG1 al AG11 y AGBI de *R. solani* usando las secuencias del ITS del ADN ribosomal e incluyendo varias réplicas. Posteriormente se seleccionaron aislamientos representativos de cada grupo anastomósico y se obtuvieron secuencias parciales del LSU del ADN ribosomal y del gen beta-tubulina. Con los datos combinados y recodificando las indels con diferentes estrategias se realizaron análisis de sensibilidad de las topologías observando resolución, número de árboles, congruencia de los caracteres e índices de apoyo.

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Capítulo I

ESTADO ACTUAL DE LA TAXONOMÍA DE *RHIZOCTONIA SOLANI* KÜHN

González-Hernández D. 2002. Revista Mexicana de Fitopatología. 20 (2): 200-205

Estado Actual de la Taxonomía de *Rhizoctonia solani* Kühn

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Resumen.
González-Hernández, D. 2002. Estado actual de la taxonomía de Rhizoctonia solani Kühn. Revista Mexicana de Fitopatología 20:200-205.
Con base en la variación fenotípica entre los aislamientos de Rhizoctonia solani y la reacción de anastomosis de hifas se ha dividido a este hongo en 14 grupos anastomósicos y varios subgrupos. Para resolver si cada grupo anastomósico corresponde a una especie distinta o a poblaciones divergentes de una sola especie se han usado caracteres morfológicos, bioquímicos, citológicos y de ultra-estructura, sin que a la fecha se haya solucionado este problema taxonómico. No obstante, estudios filogenéticos recientes con secuencias del ADN ribosomal nuclear (ITS y 28S) confirman que R. solani es un complejo de especies, por lo que varios grupos anastomósicos se deben reconocer con este rango taxonómico.

Palabras clave adicionales: sistemática, filogenia, grupos anastomósicos, ADNr.

Abstract. Based on phenotypic variation among Rhizoctonia solani isolates and hyphal anastomosis criteria, this fungus has been divided into 14 anastomosis groups and several subgroups. To solve the question concerning whether each anastomosis group corresponds to a distinct species or to divergent populations from a single species, several characters such as morphology, biochemistry, cytology, and ultrastructure have been used without solving this taxonomic problem. Nevertheless, recent phylogenetic studies using nuclear ribosomal DNA (ITS and 28S) have confirmed that R. solani is a species complex; therefore, several anastomosis groups should be recognized with this taxonomic rank.

Additional keywords: systematics, phylogeny, anastomosis groups, rDNA.

Rhizoctonia solani Kühn es un fitopatógeno importante. Se distribuye en todo el mundo causando diversas enfermedades en una gran variedad de cultivos (Parmeter, 1970; Sneh et al., 1991; Sneh et al., 1996). Además, se encuentra como saprófito y en simbiosis con orquídeas (Warcup y Talbot, 1962; Currah, 1987; Sneh et al., 1991; Pope y Carter, 2001). Por su versatilidad, ha sido objeto de múltiples estudios de tipo ecológico, patológico y de control biológico (Sneh et al., 1996). Sin embargo, su taxonomía todavía es confusa y controversial. Esto se debe a que su morfología es muy sencilla y los rasgos fisiológicos y patológicos que se usan en su clasificación varían mucho, lo cual dificulta aún más la delimitación taxonómica del grupo (Parmeter y Whitney, 1970). Otro factor que contribuye a la confusión en su taxonomía, es que raramente produce estructuras sexuales, utilizadas tradicionalmente en la taxonomía de hongos. A pesar de la diversidad de formas que presenta, R. solani se ha asignado a Thanatephorus cucumeris (Frank) Donk como el estado teleomórfico común a la mayoría de los grupos anastomósicos (AG, por sus siglas en inglés). Sin embargo, la presencia de estos grupos relativamente definidos ha hecho difícil juzgar si éstos son poblaciones divergentes de una especie o si son especies distintas. El objetivo de esta revisión es presentar los avances que se han hecho para clasificar a R. solani y resaltar la utilidad de los caracteres moleculares cuando los morfológicos son escasos o muy variables.

Enfoques tradicionales en la clasificación de Rhizoctonia solani. La clasificación es una disciplina que permite organizar la diversidad biológica, y uno de sus objetivos es integrar la biología básica y aplicada. Desde el punto de vista práctico, la clasificación de R. solani basada en sus relaciones históricas permitiría generar un marco previsible para el manejo de este hongo. Hasta mediados de los años 80's la clasificación de R. solani se basaba en rasgos morfológicos, el número de núcleos, la apariencia de los cultivos, la patología y sobre todo la anastomosis de hifas (Parmeter, 1970). Sin embargo, no se encontró una característica o combinación de éstas que sirviera para delimitar a los aislamientos de este hongo (Parmeter y Whitney, 1970). En la actualidad, se ha incorporado el microscopio electrónico de barrido con filamento de emisión de campo para observar los detalles morfológicos de las hifas (Andersen, 1996; Moore, 1996), aunque esta técnica todavía no se aplica extensivamente a diversos aislamientos. Hasta ahora, la anastomosis de hifas es el criterio más usado para clasificar a este hongo en grupos relativamente homogéneos.

Anastomosis de hifas. El sistema de clasificación fundamental para R. solani está basado en la anastomosis de las hifas. Los primeros registros de reacciones de anastomosis en ese hongo los hicieron Matsumoto (1921) y Matsumoto et al. (1931),

citados por Carling (1996); desde entonces se ha convertido en la información clave para agrupar a *R. solani*. Las hifas de los aislamientos que pertenecen a distintos grupos anastomósicos, usualmente no se fusionan, mientras que los aislamientos que pertenecen al mismo grupo anastomósico sí. No obstante, el agrupamiento por reacción de anastomosis no es completamente satisfactorio debido a que en ocasiones un aislamiento se anastomosa, total o parcialmente, con aislamientos de más de un grupo anastomósico. Además, algunos aislamientos ocasionalmente pierden su habilidad de fusionarse (Anderson, 1982, 1984; Kim y Yoshino, 1988; Vilgalys y Cubeta, 1994; Carling, 1996). Con base en el criterio de anastomosis, *R. solani* se ha dividido en 14 grupos anastomósicos (1 al 13 y el AGBI); sin embargo, se han generado subgrupos para explicar las diferencias que existen en morfología, patología, requerimiento de tiamina y grado de anastomosis (Ogoshi, 1987, 1996; Carling, 1996). Recientemente, se han propuesto categorías adicionales (C3, C2, C1 y C0) para describir las reacciones de anastomosis. Estas categorías toman en consideración los cambios citológicos que se llevan a cabo en la zona de anastomosis entre las dos hifas, además de la reacción (MacNish *et al.*, 1993; Carling, 1996). Aunque el criterio de anastomosis ha servido para agrupar a los aislamientos de *R. solani*, la relación entre el sistema de grupos anastomósicos y sus subgrupos respecto a un sistema taxonómico de especies aún no se establece.

Enfoques recientes en la clasificación de *R. solani*. Los enfoques recientes para agrupar los distintos aislamientos de *R. solani*, incorporan nuevos caracteres que se obtienen a través de técnicas bioquímicas y moleculares. Entre los caracteres bioquímicos se encuentran: la identificación de proteínas solubles, isoenzimas, lectinas, ácidos grasos y anticuerpos poli y monoclonales (Reynolds *et al.*, 1983; Kellens y Peumans, 1991; Johnk y Jones, 1993; Laroche *et al.*, 1992; Balali *et al.*, 1996; Cubeta *et al.*, 1996). Entre los moleculares están: la hibridación de ADN, los fragmentos de restricción de longitud polimórfica (RFLPs), el ADN polimórfico amplificado al azar (RAPDs) y las secuencias de ADN (Neate *et al.*, 1988; Carling y Kuninaga, 1990; Vilgalys y González, 1990; Duncan *et al.*, 1993; O'Brien, 1994; Liu *et al.*, 1993; 1995; Cubeta y Vilgalys, 1997). Sin embargo, la mayoría de estos trabajos no están enfocados a conocer las relaciones filogenéticas de *R. solani*, ya que usan estos caracteres únicamente para identificar nuevos aislamientos; para detectar subgrupos o poblaciones; o para determinar la variación genética dentro y entre grupos anastomósicos. Sólo en pocos trabajos se han utilizado caracteres bioquímicos o moleculares para clasificar a este hongo.

Caracteres bioquímicos. En tan sólo un trabajo taxonómico se empleó la asimilación de vitaminas y algunos rasgos fisiológicos para clasificar a *R. solani* y a otras especies de *Rhizoctonia* que forman simbiosis con orquídeas (Mordue *et al.*, 1989). Se determinaron caracteres del crecimiento en distintos medios de cultivo, actividad lipolítica y pruebas para algunas enzimas como fenol-oxidasa, tirosinasa y beta-galactosidasa en seis grupos anastomósicos y seis subgrupos. Los datos se analizaron a través de métodos fenéticos, elaborando matrices de similitud usando el coeficiente de Jaccard's y el coeficiente de distancia promedio; sin embargo, no se menciona el algoritmo de agrupación usado para generar el fenograma. Mordue y colaboradores (1989) concluyen que sus resultados apoyan sólo el reconocimiento del grupo anastomósico 4 como una especie distinta (*Thanatephorus praticola*). El resto de los grupos anastomósicos se mantuvieron como *T. cucumeris*.

Caracteres moleculares. Los trabajos taxonómicos con datos moleculares también son escasos (González, 1992; Kuninaga *et al.*, 1997; Boidin, 1998). González (1992) utilizó caracteres de mapas de sitios de restricción y secuencias de la terminación 5' de la subunidad larga del ADN ribosomal nuclear. Los caracteres obtenidos se utilizaron para conocer las relaciones filogenéticas de 10 grupos anastomósicos y subgrupos de *R. solani*. En este trabajo filogenético se utilizaron dos grupos externos para polarizar el cladograma (*R. cerealis* y *R. zeae*). Los análisis cladísticos mostraron que las relaciones encontradas, con pocas excepciones, son congruentes con las agrupaciones inferidas por morfología y por las reacciones de anastomosis. Sin embargo, a los grupos reconocidos tampoco se les asignó una categoría taxonómica formal, ya que los clados resultantes fueron poco robustos. Esto se debió básicamente a que la subunidad larga del ADN ribosomal en este hongo tiene muy pocos caracteres informativos. En este trabajo, también se observó de una forma preliminar, que la información filogenética en *R. solani* estaba restringida a la región de los espaciadores intergénicos (ITS, por sus siglas en inglés) 1 y 2. Consecuentemente, Kuninaga y colaboradores (1997) utilizaron las secuencias de la región del ITS 1 y 2 para conocer la variación de diez de los catorce grupos anastomósicos y algunos subgrupos de *R. solani*. En sus resultados indican que esta región es útil para identificar subgrupos con "significado biológico"; sin embargo, consideran que no es apropiada para estudios filogenéticos en *R. solani* por su elevada tasa de mutación. El método de análisis que usaron fue fenético basado en una matriz de distancias calculadas con el modelo de dos parámetros de Kimura. El fenograma se generó con el algoritmo "neighbor-joining" (union de vecinos más cercanos) (Felsenstein, 1991), y se evaluó haciendo re-muestreo con reemplazo ("bootstrap"). Sin embargo, tampoco hicieron decisiones taxonómicas formales sobre los grupos que se generaron. Recientemente, Boidin (1998) usó 104 secuencias publicadas por varios autores y disponibles en GenBank (www.ncbi.nlm.nih.gov) de la región del ITS 1 y 2 de varios aislamientos de los grupos y subgrupos anastomósicos (con excepción del AG 10 y 11), y de los géneros *Ceratobasidium*, *Waitea* y *Uthatobasidium*. Este autor también usó métodos fenéticos para el análisis de las secuencias.

El algoritmo de agrupamiento empleado fue el

"neighbor-joining", PHYLIP versión 3.4 (Felsenstein, 1991), y sus resultados estuvieron en concordancia con los del análisis cladístico de González (1992). Con esta base, Boidin (1998) les dio categoría taxonómica a los distintos aislamientos de Rhizoctonia solani y reconoció cuatro especies: Thanatephorus microesclerotius, T. sasaki, T. praticola y T. cucumeris. Los aislamientos del grupo anastomósico 1 subgrupo IB los asignó a T. microesclerotius; los del grupo anastomósico 1 subgrupos IA y IC a T. sasaki; los del grupo anastomósico 4 a T. praticola. Los de los grupos anastomósicos 2, 3, 5, 6, 8, 9 y BI a T. cucumeris. Al grupo anastomósico 7 no se le asignó categoría taxonómica. Aunque los resultados concuerdan con los obtenidos por González (1992), las conclusiones taxonómicas no están sustentadas sólidamente. La búsqueda de árboles por métodos fenéticos mediante matrices de distancias es la más sencilla, pero agrupa los taxa por su similitud total (sinapomorfias, homoplasia y simplesiomorfias combinadas) y no por sus relaciones filogenéticas. Además, el convertir las secuencias en distancias ocasiona la pérdida de la información que aportan los caracteres, por lo que los árboles basados en distancias no permiten evaluar el papel de cada carácter en la formación de los grupos. Por lo anterior, se concluye que son muy pocos los estudios taxonómicos que existen con R. solani y además, la mayoría son fenéticos.

Relaciones filogenéticas de R. solani basadas en las secuencias del ADN ribosomal nuclear. Las secuencias de ácidos nucléicos se están usando para estudiar la taxonomía de taxa cuya clasificación es incierta debido a la ausencia o plasticidad de los caracteres morfológicos (Bruns et al., 1991; Taylor, 1995). Entre los genes que se usan para estudios taxonómicos destacan los que codifican para el ARN ribosomal. Esto se debe a que un gen contiene regiones con distintas tasas de mutación. Por ejemplo, las regiones de ADN que codifican para la subunidad corta (18S) y larga (28S) del ARN ribosomal son potencialmente informativas en grupos taxonómicos de divergencia antigua. Al mismo tiempo, las regiones no codificadoras de ARN, como el ITS 1 y 2, son útiles a niveles taxonómicos de divergencia reciente (Fig. 1) (Olsen et al., 1986; Hillis y Dixon, 1991; Bowman et al., 1992; Hamby y Zimmer, 1992). Últimamente, se propuso una hipótesis de relaciones filogenéticas en R. solani, basada en un análisis cladístico con las secuencias de la región del ITS y parte del 28S del ADN ribosomal (González et al., 2001). En ese estudio se incluyeron secuencias de aislamientos colectados en doce países (Japón, Canadá, Estados Unidos, España, Australia, Costa de Marfil, Escocia, Malasia, Israel, Filipinas, Vietnam y China). Un primer análisis se realizó con 122 secuencias, de las cuales, 101 corresponden a doce de los catorce grupos anastomósicos y subgrupos (por lo menos con un duplicado). También se incluyeron 21 aislamientos de R. cerealis, un hongo binucleado, como posible grupo externo para polarizar el cladograma. Un segundo análisis se realizó adicionando secuencias de la subunidad larga (28S) del ADN ribosomal, pero con una muestra menor de aislamientos. El análisis filogenético de las secuencias se llevó a cabo con el programa PAUP* ver. 4 usando máxima parsimonia (Swofford, 2000). Debido a que el número de 122 OTUs (Operational Taxonomic Units) de la matriz de secuencias de la región del ITS, limita las habilidades de cualquier algoritmo para examinar las relaciones filogenéticas, se usaron las estrategias de búsquedas encadenadas sugeridas por Soltis y Soltis (1996). El apoyo de los clados dentro del cladograma se estimó con operaciones estadísticas de re-muestreo con reemplazo para calcular la frecuencia con la que se detecta cada clado en las búsquedas replicadas (Felsenstein, 1985). Los resultados más evidentes que se observaron en el cladograma de consenso para 21763 árboles (Fig. 2) fueron: 1) las secuencias del ITS no dieron resolución en ramas basales del cladograma, y 2) R. solani no constituye un grupo monofilético. La resolución pobre en las ramas basales se debe a la gran cantidad de topologías en competencia (21763) que se obtuvieron en estos análisis. Se observa también que los aislamientos de R. solani y R. cerealis están mezclados y dispersos a través de los clados más pequeños indicando que estas especies no son monofiléticas. Sin embargo, hay clados internos robustos que incluyen únicamente aislamientos de R. solani del mismo grupo anastomósico. Los clados que se recobran con valores por arriba del 80% de apoyo son el AG4, AG5, AG6, AG7, AG8, AG11, AGBI y los subgrupos IA, IB, IC del AG1. Esto pudiera ser una indicación de que sólo estos grupos anastomósicos son grupos naturales. El resto de los clados tienen poco apoyo. Los resultados que se obtuvieron con un número menor de muestras, pero con más caracteres son consistentes con los obtenidos para 122 aislamientos. Por lo tanto, se requiere ampliar el muestreo incluyendo más aislamientos y más caracteres, sobre todo,

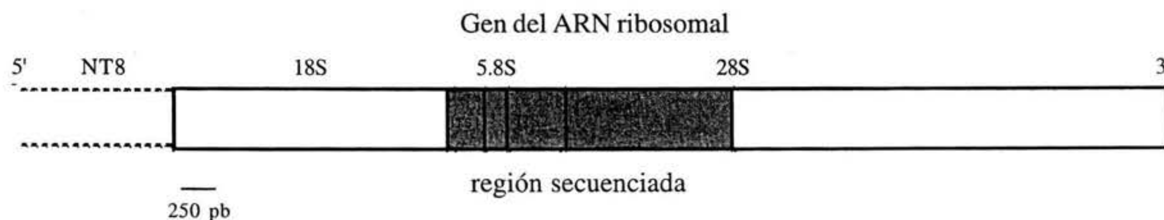

Fig. 1. Diagrama representando la región secuenciada del ITS y del 28S del ADN ribosomal nuclear (Aproximadamente 2000 nucleótidos).

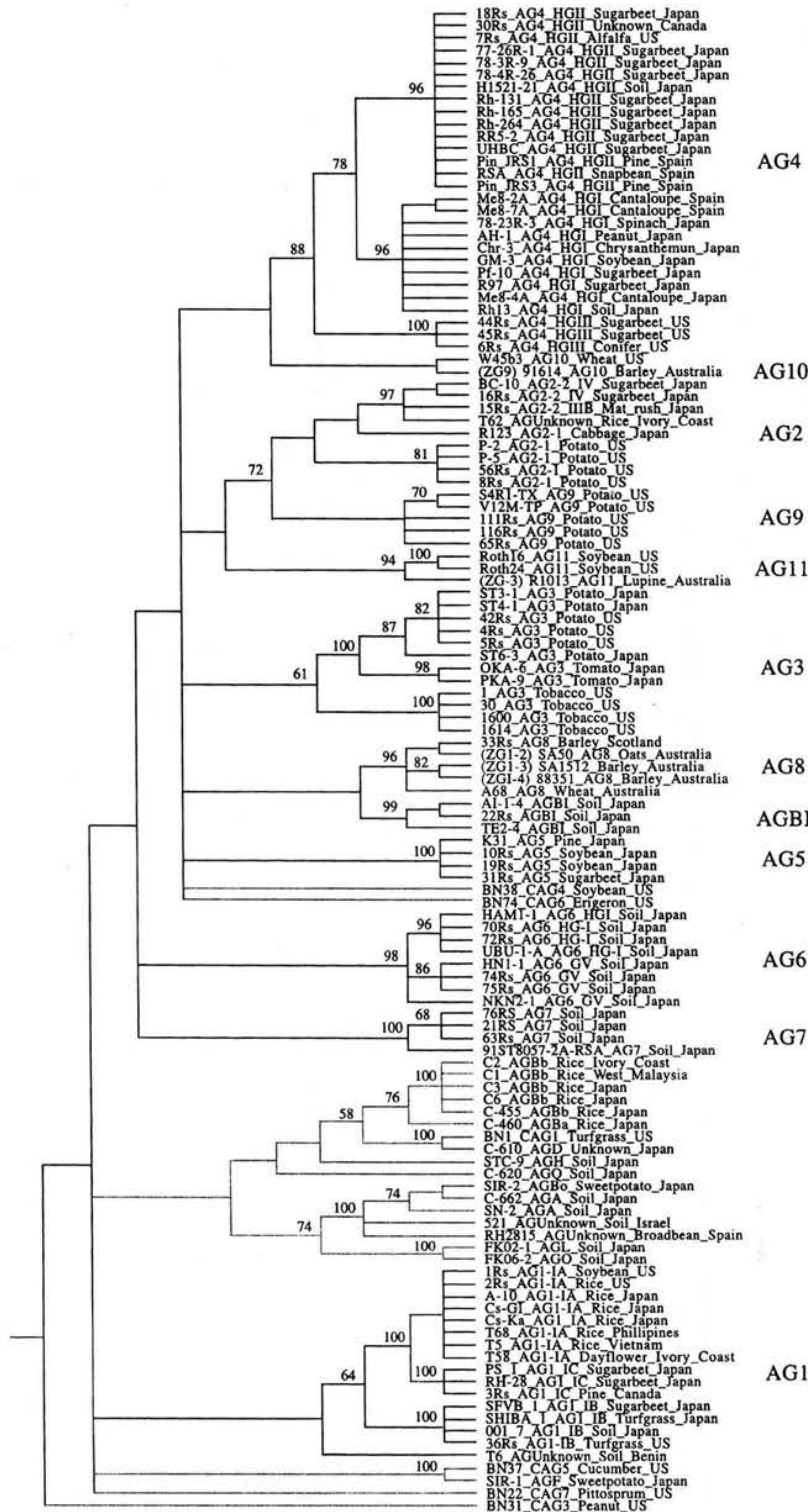

Fig. 2. Árbol de consenso basado en el análisis filogenético de las secuencias del ITS del ADN ribosomal nuclear para 122 aislamientos de *Rhizoctonia* [longitud 535, índice de consistencia (CI) = 0.551, índice de retención (RI) = 0.872]. Sobre las ramas se indica el valor relativo de apoyo a los clado por el análisis de haciendo re-muestreo con reemplazo ("bootstrap"). Las ramas en gris corresponden a los aislamientos de *R. cerealis* usados como grupo externo para polarizar el cladograma.

aquéllos que pudieran dar resolución en las ramas basales del cladograma; por ejemplo, un gen que tuviera una tasa de mutación intermedia entre el ITS y el 28S. También, se necesita identificar el grupo hermano de estos hongos. Los resultados indicaron que las especies de *Rhizoctonia* multinucleadas y binucleadas están más relacionadas de lo que se había propuesto, por lo que la condición del número de núcleos en las hifas no es un carácter diagnóstico para el reconocimiento de las distintas especies dentro del género *Rhizoctonia*. Los resultados obtenidos por González *et al.* (2001) muestran la necesidad de cambiar el sistema de clasificación actual de *R. solani* basado en grupos anastomósicos, ya que no refleja las relaciones filogenéticas de este importante hongo fitopatógeno. Para apoyar cualquier decisión taxonómica que se haga en la clasificación de *R. solani*, actualmente se están adicionando caracteres del gen beta-tubulina a la matriz de secuencias del ADN ribosomal (González datos no publicados), con el objetivo de definir si los grupos anastomósicos 4, 5, 6, 7, 8, 11, BI y los subgrupos IA, IB, IC del AG1 representan especies distintas.

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Capítulo II

RIBOSOMAL DNA SYSTEMATICS OF *CERATOBASIDIUM* AND *THANATEPHOPUS* WHITH *RHIZOCTONIA* ANAMORPHS

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Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs

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Abstract: The phylogenetic relationships of anastomosis groups (AG) of *Rhizoctonia* associated with *Ceratobasidium* and *Thanatephorus* teleomorphs were determined by cladistic analyses of internal transcribed spacer (ITS) and 28S large subunit (LSU) regions of nuclear-encoded ribosomal DNA (rDNA). Combined analyses of ITS and LSU rDNA sequences from 41 isolates representing 28 AG of *Ceratobasidium* and *Thanatephorus* supported at least 12 monophyletic groupings within *Ceratobasidium* and *Thanatephorus*. There was strong support for separation of *Ceratobasidium* and *Thanatephorus,* however, six sequences representing different AG of *Ceratobasidium* grouped with certain sequences within the *Thanatephorus* clade. Phylogenetic analysis of ITS sequence data from 122 isolates revealed 31 genetically distinct groups from *Thanatephorus* (21 groups) and *Ceratobasidium* (10 groups) that corresponded well with previously recognized AG or AG subgroups. Although phylogenetic analysis of ITS sequences provided evidence that several AG of *Ceratobasidium* may be more closely related with some AG from *Thanatephorus,* these relationships were not as strongly supported by bootstrap analysis.

Key Words: basidiomycetes, binucleate *Rhizoctonia,* phylogeny, *Rhizoctonia solani,* taxonomy

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INTRODUCTION

The *Rhizoctonia* species complex includes a wide array of genetically diverse basidiomycetes that are frequently associated with plants and soil. Many species of *Rhizoctonia* are economically important plant pathogens, as well as being saprophytes on decaying organic matter, whereas others are mycorrhizal symbionts of orchids and mosses (Warcup and Talbot 1966, Currah et al 1987, Carling et al 1999, Cubeta and Vilgalys 2000). Because of the high level of genetic diversity in morphology, pathology and physiology, taxonomic uncertainty still surrounds fungi classified as *Rhizoctonia.* Also, many isolates of *Rhizoctonia* do not reproduce sexually and are known only from their asexual stage (anamorph). However, when the sexual stage (teleomorph) has been observed, the potential taxonomic characters obtained from this stage are similar to each other and/or lacking (Anderson 1982, Parmeter and Whitney 1970). A number of teleomorph genera are connected with *Rhizoctonia* anamorphs, including *Botryobasidium* Donk, *Ceratobasidium* Rogers, *Thanatephorus* Donk, *Tulasnella* Schröt., *Uthatobasidium* Donk and *Waitea* Warcup & Talbot (Andersen 1996). Because a close phylogenetic relationship between *Ceratobasidium* and *Thanatephorus* has been previously suggested by other researchers based on an examination of septal pore and teleomorph characters (Andersen 1996, Moore 1996, Müller et al 1998, Talbot 1970, Tu and Kimbrough 1978), this study focused primarily on examining the molecular systematics of these two genera.

The most widely studied species of *Rhizoctonia, Rhizoctonia solani* Kühn, is associated with a teleomorph *Thanatephorus cucumeris* (Frank) Donk (Talbot 1970, Tu and Kimbrough 1978). However, most taxonomists agree that *R. solani* is not a single species but rather a species complex. Since isolates in the *R. solani* species complex are highly variable, confusion exists about how to classify isolates into groups and whether these groups might represent species or some other taxonomic rank (Talbot 1965, Parmeter and Whitney 1970, Tu and Kimbrough 1978, Ogoshi 1987).

The most useful system for classification of fungi within the *R. solani* complex is based largely on anastomosis grouping (AG) (Ogoshi 1987, Carling

1996). However, the determination of relationships based on anastomosis behavior of individual isolates has also been uncertain since this fungus can exhibit different types of hyphal fusion within the same AG (Carling 1996). Currently 14 AG of *R. solani* are recognized (AG-1 to AG-13 and AG-BI). Seven of 14 AG (AG-1, AG-2, AG-3, AG-4, AG-6, AG-8, and AG-9) have been further divided into subgroups to reflect differences observed in frequency of anastomosis, fatty acid and isozyme patterns, pathogenicity, thiamine requirement, and cultural appearance among isolates (Ogoshi 1987, Stevens-Jonks and Jones 2001). A similar classification system has been developed for *Ceratobasidium,* where seven and 19 anastomosis groups have been described from the US (CAG-1 to CAG-7) and Japan (AG-A to AG-S), respectively (Burpee et al 1980, Ogoshi 1987). One anastomosis group of *Ceratobasidium* (AG-B) has been further divided into subgroups based on cultural characteristics and frequency of anastomosis (AG-Ba, AG-Bb, and AG-Bo). Although the AG system has provided a useful criterion for characterizing *Ceratobasidium* and *Thanatephorus,* the relationship of AG and AG subgroups to species or other taxonomic units has not been formally established. At least 14 of the 26 recognized AG associated with a *Ceratobasidium* teleomorph lack a species epithet for their respective *Rhizoctonia* anamorph. The challenge of determining species or other taxonomic units is also exacerbated because 17 AG are not associated with a well-defined species of *Ceratobasidium* based on examination of morphological characters.

With the advent of DNA-based molecular techniques, there has been a resurgence of interest in *Rhizoctonia* taxonomy. Several researchers (González 1992, Boysen et al 1996, Kuninaga et al 1997, Boidin et al 1998, Johanson et al 1998, Salazar et al 1999, 2000) have examined the phylogenetic and taxonomic relationships of AG and AG subgroups of *R. solani* using sequence analysis of the internal transcribed spacer (ITS 1 and ITS 2) and 5.8S regions of nuclear-encoded ribosomal DNA (rDNA). Results from these studies have shown that there is no sequence variation in the 5.8S region, while the ITS regions display a high level of sequence variation among isolates of *Ceratobasidium* and *Thanatephorus.* Most of these studies have concluded that molecular relationships are largely congruent with relationships inferred from hyphal anastomosis reactions. However, no taxonomic decisions about the phylogenetic groupings were made in the previous studies.

In this paper molecular systematics methods were used to test the hypotheses that 1) *Ceratobasidium* and *Thanatephorus* represent distinct evolutionary lineages of fungi with *Rhizoctonia* anamorphs and 2) anastomosis groups represent the most fundamental evolutionary units within *R. solani.* The application of phylogenetic data for unambiguous identification of *Rhizoctonia* anamorphs of *Ceratobasidium* and *Thanatephorus* is also presented.

MATERIALS AND METHODS

Isolates and DNA extraction.—Sixty isolates representing 28 AG of *Ceratobasidium* and *Thanatephorus* were included in this study (TABLE I). Isolates were grown in 20 mL of potato dextrose broth (Difco) for 3–5 d at 25 C, harvested by filtration, lyophilized, ground to a fine powder in liquid nitrogen and stored at −20 C. Genomic DNA was extracted according the miniprep method of Raeder and Broda (1985). Mycelium was suspended in 500 mL of extraction buffer (15 mM NaCl, 50 mM Tris [pH 8.0], 10 mM Na_2EDTA, 1% [w/v] SDS) for 15 min. The solution was extracted with an equal volume of chloroform-isoamyl alcohol 24:1 (v/v) and centrifuged at 13 200 rpm for 15 min. The upper aqueous layer was incubated with 50 mL of RNase A (5 mg/mL) at 37 C for 30 min. The solution was re-extracted with an equal volume of chloroform-isoamyl alcohol 24:1 (v/v), and centrifuged at 13 200 rpm for 15 min. The upper aqueous layer was mixed with 0.1 volumes of 3 M sodium acetate and mixed, and 1.8 volumes of cold absolute ethanol was then added to precipitate DNA. The pellet was collected, rinsed with 70% ethanol, and dried under a vacuum.

DNA amplification and sequencing.—Prior to amplification, genomic DNA was purified on a 0.6% low-melting-point agarose gel, cut out of the gel and dissolved up to 0.001 μg per μL in distilled water. Approximately 0.01 μg of purified DNA was amplified by the polymerase chain reaction (PCR). Reactions for PCR amplification were performed in a 50 μL mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 μL of each of the four deoxynucleoside triphosphates, 5 pmol of each primer, 10 μL of template and 2.5 units of *Taq* polymerase. The amplifications were performed with a thermal cycler 480 from Perkin-Elmer (Norwalk, Connecticutt). The cycle parameters were an initial denaturation at 96 C for 5 min, followed by 25 cycles consisting of denaturation at 96 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min, and a final extension for 7 min at 72 C. The oligonucleotide primers ITS1 (or ITS5) and ITS4 (White et al 1990) were used for amplification and sequencing of the internal transcribed spacer (ITS) region of the nuclear-encoded rDNA region, while primers LROR, LR22R, LR3, and LR5 were used to amplify and sequence the 5′-portion of the large subunit (=28S RNA) rDNA region (Hopple and Vilgalys 1999).

Amplified DNA from each isolate was purified by adding 0.3 volumes of 7.5 M ammonium acetate and followed by 2.5 volumes of cold 95% ethanol. The pellet was collected, rinsed with 70% ethanol, and dried under a vacuum. Amplified DNA was sequenced using dye terminator chemistry (Applied Biosystems, Perkin Elmer) or a thermo sequenase

TABLE I. Anastomosis (AG/CAG) and subgroup designation, origin and source of isolates of *Rhizoctonia* species with *Ceratobasidum* and *Thanatephorus* teleomorphs and their GenBank accession numbers

Anastomosis group/ subgroup	Isolate	Origin (Source)[1]	Region sequenced		GenBank accession number[2]
			ITS1 and ITS2	28S	
Thanatephorus (R. solani)					
AG-1-IA	1Rs (ATCC 66159)	Soybean, US (14)	*	*	AF354060
	2Rs (ATCC 66158)	Rice, US (14)	*		AF354097
	A-10	Rice, Japan (10)	*		AB000010
	Cs-Gi	Rice, Japan (10)	*		AB000016
	Cs-Ka	Rice, Japan (10)	*		AB000017
	T68 (IMI 358761)	Rice, Philippines (8)	*		AJ000197
	T5 (IMI 360366)	Rice, Vietnam (8)	*		AJ000199
	T58 (IMI 360021)	Dayflower, Ivory Coast (8)	*		AJ000200
AG-1-IB	36Rs (ATCC 66150)	Turfgrass, US (4)	*	*	AF354059
	SFBV-1	Sugar beet, Japan (10)	*		AB000038
	001-7	Soil, Japan (10)	*		AB000025
	SHIBA-1	Turfgrass, Japan (10)	*		AB000039
AG-1-IC	3Rs (ATCC 44661)	Pine, Canada (1)	*	*	AF354058
	PS-1	Sugar beet, Japan (10)	*		AB000029
	RH-28	Sugar beet, Japan (10)	*		AB000035
AG-2-1	8Rs (ATCC 44658)	Soil, Australia (1)	*	*	AF354063
	56Rs (ATCC 62805)	Potato, US (6)	*		AF354105
	P-2	Potato, US (6)	*		AB000026
	P-5	Potato, US (6)	*		AB000027
	R123	Cabbage, Japan (10)	*		AB000030
AG-2-2 IIIB	15Rs	Mat rush, Japan (AO)	*		AF354116
AG-2-2 IV	BC-10 16Rs	Sugar beet, Japan (10)	*		AB000014
		Sugar beet, Japan (13)	*		AF354117
AG-3	4Rs (ATCC 14006)	Potato, US (12)	*	*	AF354064
	5Rs (ATCC 44660)	Potato, US (1)	*		AF354107
	42Rs (ATCC 14701)	Potato, US (2)	*		AF354106
	1	Tobacco, US (18)	*		AB000001
	30	Tobacco, US (18)	*		AB000002
	1600	Tobacco, US (18)	*		AB000004
	1614	Tobacco, US (18)	*		AB000005
	OKA-6	Tomato, Japan (10)	*		AB000023
	OKA-9	Tomato, Japan (10)	*		AB000024
	ST3-1	Potato, Japan (10)	*		AB000041
	ST4-1	Potato, Japan (10)	*		AB000042
	ST6-3	Potato, Japan (10)	*		AB000043
AG-4 HGI	AH-1 (ATCC 76126)	Peanut, Japan (10)	*	*	AB000012, AF354118
	78-23R-3	Spinach, Japan (13)	*		AB000007
	Chr-3	Chrysanthemum, Japan (13)	*		AB000015
	GM-3	Soybean, Japan (13)	*		AB000018
	Pf-10	Sugar beet, Japan (13)	*		AB000028
	R97	Sugar beet, Japan (13)	*		AB000031
	Me 8-2A	Cantaloupe, Spain (3)	*		RSU19952
	Me 8-4A (Me84)	Cantaloupe, Spain (3)	*		RSU19954
	Me 8-7A (Me87)	Cantaloupe, Spain (3)	*		RSU19956
	Rh13	Soil, Spain (3)	*		RSU19960
AG-4 HGII	Rh-165 (ATCC 76127)	Sugar beet, Japan (10)	*		AB000033
	77-26R-1	Sugar beet, Japan (13)	*		AB000006
	78-3R-9	Sugar beet, Japan (13)	*		AB000008
	78-4R-26	Sugar beet, Japan (13)	*		AB000009
	HI521-21	Soil, Japan (13)	*		AB000020

TABLE I. Continued

Anastomosis group/ subgroup	Isolate	Origin (Source)[1]	Region sequenced: ITS1 and ITS2	Region sequenced: 28S	GenBank accession number[2]
	Rh-131	Sugar beet, Japan (13)	*		AB000032
	Rh-264	Sugar beet, Japan (13)	*		AB000034
	RR5-2	Sugar beet, Japan (13)	*		AB000036
	UHBC	Sugar beet, Japan (13)	*		AB000045
	Pin JRS1	Pine, Spain (3)	*		RSU19958
	Pin JRS3	Pine, Spain (3)	*		RSU19959
	RSA	Snapbean, Spain (3)	*		RSU19964
	7Rs (ATCC 44662)	Alfalfa, US (1)	*	*	AF354074
	18Rs	Sugar beet, Japan (13)	*	*	AF354072
	30Rs (ATCC 48803)	Unknown, Canada (5)	*	*	AF354073
AG-4 HGIII	6Rs (ATCC 42127)	Conifer, US (5)	*	*	AF354077
	44Rs (ATCC 14007)	Sugar beet, US (12)	*	*	AF354075
	45Rs (ATCC 10177)	Sugar beet, US (9)	*	*	AF354076
AG-5	10Rs	Soybean, Japan (13)	*	*	AF354078
	19Rs	Soybean, Japan (13)	*		AF354112
	31RS	Sugar beet, Japan (15)	*		AF354113
	K31	Pine, Japan (13)	*		AB000021
AG-6 HG-I	72Rs	Soil, Japan (13)	*	*	AF354061
	70Rs	Soil, Japan (13)	*		AF354102
	UBU-1-A	Soil, Japan (13)	*		AF354103
	HAM1-1	Soil, Japan (13)	*		AB000019
AG-6 GV	74Rs	Soil, Japan (13)	*	*	AF354062
	75Rs	Soil, Japan (13)	*		AF354104
	HN1-1	Soil, Japan (13)	*		AF354101
	NKN2-1	Soil, Japan (10)	*		AB000022
AG-7	76Rs	Soil, Japan (6)	*	*	AB000003, AF354096
	63Rs	Soil, Japan (6)	*		AF354099
	21RS	Soil, Japan (6)	*		AF354098
	91ST8057-2A-RSA	Soil, US (17)	*		AF354100
AG-8	33Rs	Barley, Scotland (4)	*	*	AF354066
	(ZG1-2)SA50	Oats, Australia (7)	*	*	AF354067
	(ZG1-3)SA1512	Barley, Australia (16)	*	*	AF354068
	(ZG1-4)88351	Barley, Australia (11)	*	*	AF354069
	A68	Wheat, Australia (11)	*	*	AB000011, AF354119
AG-9	65Rs (ATCC 62804)	Potato, US (6)	*		AF354109
	111Rs	Potato, US (6)	*		AF354108
	116Rs	Potato, US (6)	*	*	AF354065
	S4R1-TX	Potato, US (6)	*		AB000037
	V12M-TP	Potato, US (6)	*		AB000046
AG-10	W45b3	Wheat, US (13)	*		AF354111
	(ZG9)91614	Barley, Australia (11)	*	*	AF354071
AG-11	(ZG-3)R1013	Lupine, Australia (20)	*	*	AF354079
	Roth16	Soybean, US (17)	*		AF354114
	Roth24	Soybean, US (17)	*		AF354115
AG-BI	22Rs	Soil, Japan (13)	*	*	AF354070
	AII-4	Soil, Japan (13)	*		AF354110
	TE2-4	Soil, Japan (13)	*		AB000044
Unknown	T62 (IMI 360038)	Rice, Ivory Coast (8)	*		AJ000201
	T6 (IMI 369673)	Soil, Benin (8)	*		AJ000202
Ceratobasidium (binucleate *Rhizoctonia* spp.)					
AG-A	C-662	Soil, Japan (13)	*	*	AF354092
	SN-2	Soil, Japan (13)	*		AB000040
AG-Ba	C-460	Rice, Japan (13)	*	*	AF354088

TABLE I. Continued

Anastomosis group/ subgroup	Isolate	Origin (Source)[1]	Region sequenced: ITS1 and ITS2	Region sequenced: 28S	GenBank accession number[2]
AG-Bb	C-455	Rice, Japan (13)	*	*	AF354087
	C2 (IMI 375129)	Rice, Ivory Coast (8)	*		AJ000191
	C1 (IMI 062599)	Rice, West Malaysia (8)	*		AJ000192
	C3 (IMI 375130)	Rice, Japan (8)	*		AJ000193
	C6 (IMI 375133)	Rice, Japan (8)	*		AJ000194
AG-Bo	SIR-2	Sweet potato, Japan (13)	*	*	AF354091
AG-D	C-610	Unknown, Japan (13)	*	*	AF354090
AG-F	SIR-1	Sweet potato, Japan (13)	*	*	AF354085
AG-H	STC-9	Soil, Japan (13)	*	*	AF354089
AG-L	FK02-1	Soil, Japan (13)	*	*	AF354093
AG-O	FK06-2	Soil, Japan (13)	*	*	AF354094
AG-Q	C-620	Soil, Japan (13)	*	*	AF354095
CAG-1	BN1	Turfgrass, US (4)	*	*	AF354086
CAG-3	BN31	Peanut, US (4)	*	*	AF354080
CAG-4	BN38	Soybean, US (4)	*	*	AF354081
CAG-5	BN37	Cucumber, US (4)	*	*	AF354082
CAG-6	BN74 (ATCC 13247)	*Erigeron*, US (4)	*	*	AF354083
CAG-7	BN22 (FL FTCC585)	*Pittosporum*, US (4)	*	*	AF354084
Unknown	Rh2815	Broadbean, Spain (19)	*		RSU19962
	521 (RH2815L)	Soil, Israel (19)	*		RSU19963

[1] Isolates provided by; 1 = N. Anderson; 2 = K. Barker; 3 = M. Boysen; 4 = L. Burpee; 5 = E. Butler; 6 = D. Carling; 7 = A. Dube; 8 = A. Johannson; 9 = J. Kotila; 10 = S. Kuninaga; 11 = G. MacNish; 12 = G. Papavizas; 13 = A. Ogoshi; 14 = N. O'Neill; 15 = S. Naito; 16 = S. Neate; 17 = C. Rothrock; 18 = D. Shew; 19 = B. Sneh; and 20 = M. Sweetingham.

[2] GenBank numbers with an "AF" prefix represent isolates sequenced in this study.

dye terminator cycle sequencing pre-mix kit (Amersham Life Science) as described by the manufacturer. The sequencing products were separated in a 6% polyacrylamide gel using an ABI-373A automated sequencer (Perkin-Elmer, Foster City, California).

Data analyses.—Two aligned sequence data sets were developed for phylogenetic analyses. The first data set included 41 rDNA sequences spanning both ITS and adjacent 5′-end LSU regions. The second data set included 122 ITS sequences (40 sequences from the first data set, 19 new sequences from this study and 63 previously characterized sequences from GenBank) (Boysen et al 1996, Johanson et al 1998, Kuninaga et al 1997). The complete list of isolates, sequences, and GenBank deposition numbers is presented in TABLE I.

For each data set, sequences were aligned using the Clustal V program (Higgins et al 1992) within the Megalign computer software package (Lasergene, DNASTAR Inc.) and later adjusted by visual examination. Regions of ambiguous alignment were excluded from further phylogenetic analysis. Other regions containing single-nucleotide insertions or deletions were included in the phylogenetic analyses, with gaps treated as missing data. The final data sets used for phylogenetic analysis are available from the authors and have also been deposited with TreeBASE (http://herbaria.harvard.edu/treebase/, number SN931).

Phylogenetic analysis was performed using the maximum parsimony criterion in PAUP* (Swofford 2000). Because both data sets were too large to perform complete tree-searches, alternative search strategies which maximized the exploration of larger "tree-space" (Maddison et al 1992, Olmstead et al 1993, Moncalvo et al 2000, Soltis et al 1998) were employed. This approach included full heuristic searches with simple taxon addition sequences when possible (with TBR swapping and MAXTREES unlimited), as well as multiple searches using random addition sequences, NNI swapping and MAXTREES set to between 2–10 trees at each step. Branch support was assessed by bootstrap analysis (Felsenstein 1985) based on 500 replicate heuristic searches using the "fast bootstrap" option in PAUP*. Because appropriate outgroups were not available, all phylogenies were midpoint rooted. An initial attempt included *Tulasnella arinosa* and *Botryobasidium intertexum* as potential outgroups, but sequence alignment was problematic due to low similarity and ambiguous correspondence of nucleotides. Additional tree topologies were also evaluated by constraining phylogenetic searches to seek trees which were consistent with alternative taxonomic hypotheses and then testing these against the most-parsimonious trees using Templeton's nonparametric test in PAUP*. Genetic divergence between ITS sequences were calculated in PAUP* (Swofford 2000) using Kimura's 2-parameter distance measure with base frequencies estimated from the data and gamma parameter = 0.5.

RESULTS

Combined analysis of ITS and LSU rDNA sequence data.—Alignment of the 5′ end of the LSU region was easily accomplished by visual examination and associated with the highly conserved nature of this region with most length mutations involving insertions or deletions of a single nucleotide (Hopple and Vilgalys 1999). In contrast to the LSU region, the ITS regions (particularly ITS1) contained many small deletions and insertions of one to several nucleotides, or were difficult to align. These variable regions did not align among isolates but were specific for certain AG subgroups of *Thanatephorus*. The combined ITS and LSU data set consisted of at total of 1643 aligned nucleotide positions, which was reduced to 1517 positions after removal of regions with ambiguous alignment.

Heuristic searches revealed three most-parsimonious trees with a length of 2130, CI = 0.483, and RI = 0.71; one of the most parsimonious trees is shown in FIG. 1. A strict consensus of all three trees differed only in the placement of several unsupported branches. Phylogenetic analysis of the combined ITS and LSU data set supported several monophyletic groupings as follows: within *Ceratobasidium* (FIG. 1), AG-Ba with AG-Bb (clade 1); AGL with AG-O (clade 2); AG-A with AG-Bo (clade 3); AG-D with CAG-1 (clade 4); and AG-F with CAG-5 (clade 7); and within *Thanatephorus*, AG-4 HGI and AG-4 HGII (clade 5); AG-4 HG III (clade-6); AG-6 HGI and AG-6 GV (clade 8); AG-1-IA and AG-1-IC (clade 9); AG-2-1 and AG-9 (clade 10); AG-8 (clade 11); and AG-5 with AG-11 (clade 12).

Within the combined ITS and LSU tree, there was strong support (93% bootstrap value) for separation of two major groups of sequences corresponding largely but not completely with taxonomic division of isolates according to their associated teleomorph (*Ceratobasidium* italicized, and *Thanatephorus* not italicized). However, six sequences representing *Ceratobasidium* anastomosis groups CAG-3, CAG-4, CAG-5, CAG-6, CAG-7, and AG-F were found to group with other sequences belonging to the larger *Thanatephorus* clade. Phylogenetic searches in which *Ceratobasidium* and *Thanatephorus* were constrained to be monophyletic revealed trees that were significantly longer (22 additional steps) than the most parsimonious trees when tested using the Templeton test ($p < 0.02$). Based on combined ITS and LSU sequences, we can reject the hypothesis that *Ceratobasidium* and *Thanatephorus* as morphologically circumscribed represent mutually monophyletic groups.

Analysis of ITS rDNA sequence data.—The ITS data matrix included 853 aligned positions, of which 270 positions had to be excluded from analysis because they could not be unambiguously aligned. Of the remaining 583 positions, 162 were phylogenetically informative. Parsimony analysis yielded over 5000 equally parsimonious trees with a length of 535 (CI = 0.551, RI = 0.872). One of the most parsimonious trees is shown as a phylogram in FIG. 2 together with fast bootstrap support values for branches with support.

Phylogenetic analysis of the ITS sequence data revealed numerous well supported terminal groupings (bootstrap support = 72–100%) that correspond with previously recognized AG or AG subgroups within *Thanatephorus* (AG-1-IA and AG-1-IC, AG-1-IB; AG-6 HGI and AG-6 GV; AG-2-1 and AG-9; AG-4 HGI and AG-4 HGII, AG-4 HGIII; and AG-3; AG-5; AG-7; AG-8; AG-10; AG-11; AG-BI) and *Ceratobasidium* (AG-F and CAG-5; AG-L and AG-O; CAG-1 and AG-D; and AG-Ba and AG-Bb) (FIG. 2). At least 31 genetically distinct groupings could be identified based on differences in their ITS sequences from *Thanatephorus* (21 groups) and *Ceratobasidium* (10 groups). Within any single genetic group, ITS sequences showed little or no genetic divergence. Within an AG, sequence divergence among strains from the same AG varied as follows: AG-1 (from 2–4%), AG-2 (1.4%–3.5%), AG-4 (1–3%) and AG-6 (0–0.5%). Genetic divergence between unrelated groups of AG within *Thanatephorus* varied more widely, from 0.9% (between AG-2-1 and AG-9) to 8.1% (between AG-1-IA and AG-BI). Although sampling within *Ceratobasidium* was more limited, sequence divergence among genetic groups of *Ceratobasidium* appears to be greater than within *Thanatephorus*, ranging from 1.1% (between AG-Bo and AG-A) up to 16.1% (CAG-1 and CAG-3). The highest levels of sequence divergence were observed between isolates of *Thanatephorus* and *Ceratobasidium*, ranging from 1.9% between CAG-6 and AG-9, up to 16.5% between AG-1-IB and CAG-1.

Phylogenetic analysis of ITS sequences also suggested that several *Ceratobasidium* anastomosis groups might be more closely related with other groups from *Thanatephorus* than with other groups from *Ceratobasidium*. For example, phylogenetic analysis placed both CAG-4 and CAG-6 basal to the AG-4 clade (FIG. 2). However, these relationships are not strongly supported by bootstrap analysis and by other equally parsimonious trees. Phylogenetically constrained trees (with *Thanatephorus* and *Ceratobasidium* each constrained to be monophyletic) were not significantly longer (four extra steps) than the most parsimonious ITS trees (Templeton test, $p = 0.21–0.25$). Based on ITS evidence alone, therefore, monophyly of each teleomorph genus cannot be rejected.

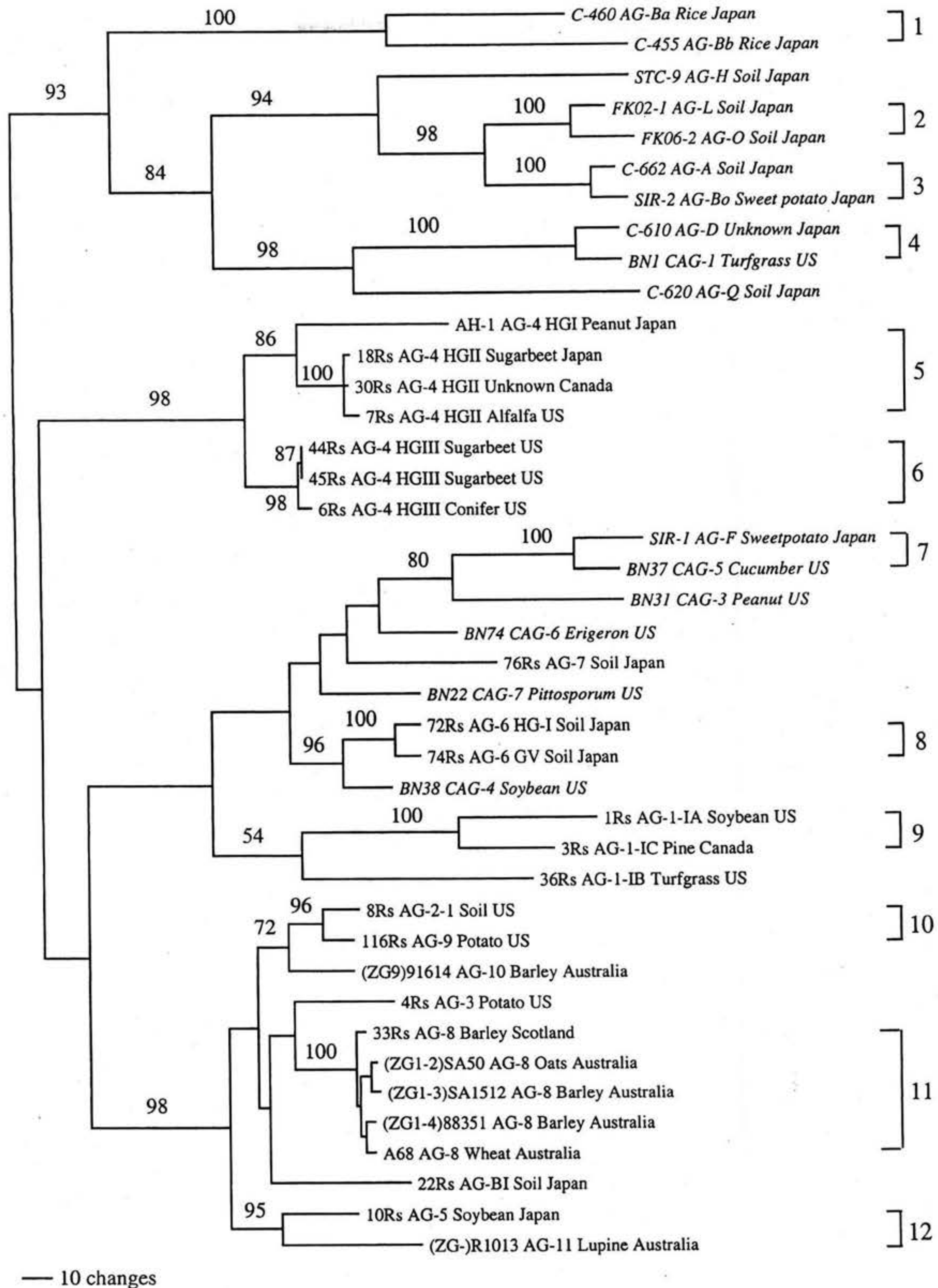

FIG. 1. One of three most parsimonious trees based on phylogenetic analyses with PAUP* Swofford 2000) of internal transcribed spacer (ITS) and adjacent 28S large subunit (LSU) regions of nuclear-encoded ribosomal DNA (rDNA). This analysis includes 41 isolates representing 28 anastomosis groups of *Ceratobasidium* and *Thanatephorus*. Tree length is 2130 steps with 462 phylogenetically informative characters. Consistency index (CI) = 0.483, and retention index (RI) = 0.71. The relative support for each clade is indicated by bootstrap values on branches.

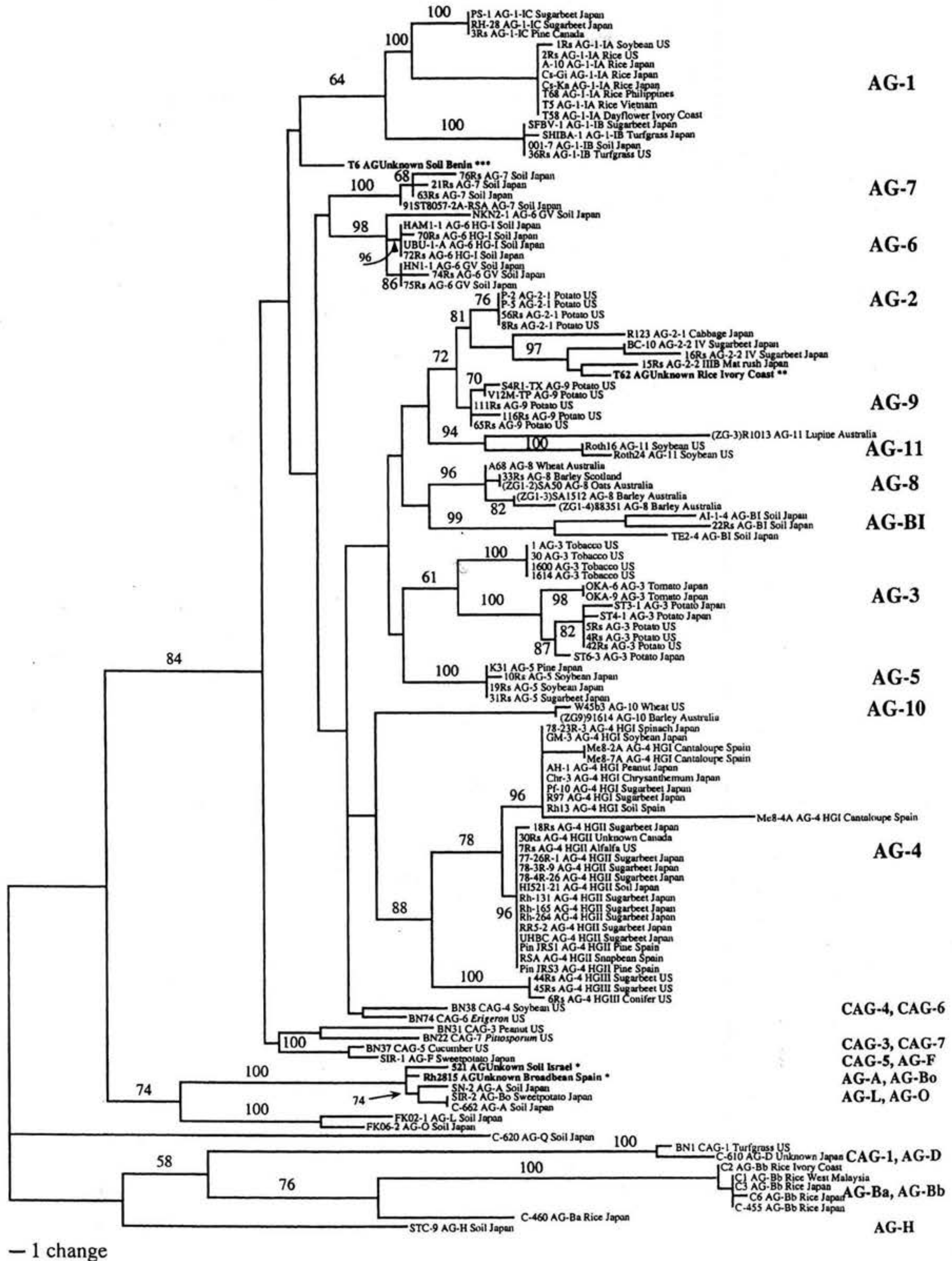

FIG. 2. Consensus tree based on phylogenetic analyses with PAUP* (Swofford 2000) of internal transcribed spacer (ITS) regions of nuclear-encoded ribosomal DNA (rDNA) from 122 isolates representing 28 anastomosis groups of *Ceratobasidium* and *Thanatephorus*. Tree length is 535 steps with 162 phylogenetically informative characters. Consistency index (CI) = 0.551 and retention index (RI) = 0.872. The relative support for each clade is indicated by bootstrap values on branches.

DISCUSSION

Phylogenetic distinction between Thanatephorus *and* Ceratobasidium.—Much controversy still surrounds the taxonomy of *Ceratobasidium* and *Thanatephorus,* and their phylogenetic placement within the Basidiomycota. Results from this study (FIGS. 1 AND 2) suggest that at least some isolates of *Thanatephorus* may be more closely related with other isolates from *Ceratobasidium.* However, these relationships were not as strongly supported by bootstrap analysis. Recent molecular evidence from small subunit rDNA sequence analysis suggests that *Thanatephorus, Ceratobasidium,* and *Waitea* (of the Ceratobasidiales) are closely related to each other and to other fungi belonging to the euagaric clade, which includes mushrooms and their allies (Hibbett and Thorn 2001).

When grown on nutrient medium, many isolates of *Thanatephorus* and *Ceratobasidium* often have a similar appearance (Kotila 1929, Parmeter et al 1967, Burpee et al 1980, Ogoshi 1987). In this study, all isolates were examined for their nuclear condition and *Rhizoctonia* species associated with *Ceratobasidium* had only two nuclei per hyphal cell, whereas those associated with *Thanatephorus* were multinucleate (3 or more nuclei per hyphal cell). In addition, each isolate of the six AG that clustered within the *Thanatephorus* clade was re-examined for its nuclear condition and found to be binucleate. Burpee et al (1980) reported that isolates of binucleate *Rhizoctonia* (representing CAG-3, CAG-4 and CAG-5) caused pre-and post emergence damping-off of bean, pea and tomato, and were often morphologically indistinguishable from *R. solani.* In absence of a teleomorph, Burpee et al (1980) assigned these isolates to *Ceratobasidium* based on their hyphal anastomosis reactions and binucleate nuclear condition, but also expressed some uncertainty about their taxonomic placement. Yokoyama and Ogoshi (1986) have observed hyphal fusion among isolates of *Ceratobasidum* (AG-F) and *Thanatephorus* (AG-6) which also suggested that certain isolates of *Ceratobasidium* and *Thanatephorus* may possibly be genetically related. However, since only a single isolate for each of the six AG of *Ceratobasidium* that clustered in the *Thanatephorus* clade was sequenced, more testing with additional isolates representing each of these AG is required to substantiate these relationships.

Few studies to date have examined taxonomic relationships among *Ceratobasidium* and *Thanatephorus* using DNA-based methods. Johanson et al (1998) studied ITS sequence data and found that isolates of *Ceratobasidium oryzae-sativae* (anamorph = *R. oryzae-sativae,* anastomosis group AG-Bb) were more closely related with *T. cucumeris* (=*R. solani* AG-1) than with *Waitea circinata* (anamorph = *R. oryzae,* WAG-O). Also, Boidin et al (1998) analyzed a rather large ITS data matrix to infer phylogenetic relationships among fungi with *Rhizoctonia* anamorphs, and concluded that *Ceratobasidium* was closely related to *Thanatephorus, Uthatobasidium* and *Waitea.* Our study extends previous research by analyzing a larger set of rDNA sequences from a greater variety of both multinucleate (*Thanatephorus*) and binucleate (*Ceratobasidium*) isolates. Statistical evidence from rDNA phylogenies also suggests that some isolates currently classified in *Ceratobasidium* based on nuclear condition and hyphal anastomosis reaction might be more correctly classified within *Thanatephorus.* To better resolve this question, additional phylogenetic analyses are still needed using isolates that represent outgroup taxa. Based on molecular as well as ultrastructural evidence, possible outgroup taxa to consider include *Waitea* or *Uthatobasidium,* as well as other members of the euagaric clade in the homobasidiomycetes (Hibbett and Thorn 2001). All of these species possess variably perforated parenthosomes.

Identification of anastomosis groups, AG subgroups and species based on rDNA sequences.—Numerous studies have demonstrated the tremendous genetic diversity that exists within both *Thanatephorus* and *Ceratobasidium,* manifested as an ever growing number of genetically distinct anastomosis groups and subgroups (Vilgalys and Cubeta 1994, Carling 1996, Kuninaga et al 1997, Salazar et al 2000). As more laboratories begin to collect sequence data for different isolates of *Rhizoctonia,* analysis of rDNA sequences can serve as an independent and convenient method for identifying genetically distinct groups within the *Rhizoctonia* species complex.

Because the ITS region is known to have a higher rate of molecular evolution than other ribosomal genes (Hibbett et al 1997), ITS sequences have been particularly useful for identifying previously undetected genetic groups at the species level. In a recent study, Kuninaga et al (1997) demonstrated the utility of rDNA ITS sequences for assessing genetic diversity and identification of AG and AG subgroups among 45 isolates of *Thanatephorus.* Isolates belonging within the same AG shared high sequence similarity (above 96%), whereas isolates from different AG showed significantly less similarity (55% or higher). In this study, we combined data primarily from the previous work of Kuninaga et al (1997) with new sequences from our lab and with sequences from other studies (Boysen et al 1996, Johanson et al 1998). For the 99 *Thanatephorus* isolates included in this study, at least 21 genetically distinct ITS groups could be identified. These 21 groups corresponded extremely

well with known groups based on hyphal anastomosis typing. In addition, at least 10 putative genetic groups were evident for 23 isolates of *Ceratobasidium* based on their ITS rDNA sequences. Interestingly, although fewer isolates of *Ceratobasidium* were investigated in our study, their genetic diversity was higher than for isolates of *Thanatephorus*. This observation suggests that *Ceratobasidium* may also harbor many additional as-yet-undescribed genetic groups.

In general, analysis of ITS and combined ITS and LSU sequence data provided similar conclusions about relationships of *Rhizoctonia* anamorphs of *Ceratobasidium*, as have been described in a previous studies based on RFLP analysis of LSU (Cubeta et al 1991). For example, AG-A and AG-Bo (*C. cornigerum*); AG-Ba (*C. setariae*, anamorph = *R. fumigata*) and AG-Bb (*C. oryzae-sativae*); AG-D and CAG-1 (*C. cereale* Murray and Burpee (anamorph = *R. cerealis* Van der Hoeven); AG-F and CAG-5 (*Ceratobasidium* sp.); and AG-L and AG-O (*Ceratobasidium* sp.) appear to represent independent evolutionary lineages that correspond to different species of *Ceratobasidium*. Additional sampling should reveal increased resolution of phylogenetic structure in these groups and phylogenetic accuracy will improve with the addition of replicates per taxon and with an increase in the number of characters (Smouse et al 1991, Graybeal 1998, Poe 1998).

Experimental results from this and previous studies that have examined rDNA ITS sequences of *R. solani* (González 1992, Boysen et al 1996, Kuninaga et al 1997, Boidin et al 1998, Johanson et al 1998, Salazar et al 1999, 2000) have several common themes: 1) most AG and AG subgroups represent genetically distinct groups which support previous separation based on hyphal anastomosis behavior, 2) certain AG are not monophyletic; and 3) there is greater taxonomic support for AG subgroups than AG. Given the genetic diversity that has been identified within an AG, only a few studies have explored the relationship of AG and subgroups to species or other taxonomic units. Boidin et al (1998) recognized four species: 1) *Thanatephorus microsclerotius* (Weber) Boidin, Mugmier & Canales including AG-1-IB; 2) *T. sasaki* (Shirai) Tu & Kimbrough including AG-1-IA and AG-1-IC; 3) *T. praticola* (Kotila) Flentje including AG-4; and 4) *T. cucumeris* including AG-2, 3, 5, 6, 8, 9 and BI. AG-7 was not included in any of the above species. A biochemical approach was used by Mordue et al (1989) to study the taxonomy of *Rhizoctonia* (12 isolates of *R. solani* representing eight AG and 13 species of *Rhizoctonia* from orchids) based on cultural characteristics, carbon and nitrogen utilization and enzyme production. Mordue et al (1989) recognized AG-4 as a distinct species, *Thanatephorus praticola*, while the remaining subgroups of *R. solani* were assigned to *T. cucumeris*. However, they did not recognize AG-1-IA (*T. sasakii* (Shirai) Tu & Kimbrough, the causal agent of sheath blight of rice), AG-1-IB (*Corticium microsclerotium* Weber (anamorph = *Rhizoctonia microsclerotia* Matz, the causal agent of web blight of bean), or AG-1-IC as taxa distinct from *T. cucumeris*. Kuninaga et al (1997) was able to separate subgroups within *R. solani* AG-1 (IA, IB and IC) and AG-4 (HGI and HGII) based on sequence analysis of rDNA ITS region and suggested that they represent independent evolutionary units. Several groups with higher support in a neighbor joining tree were evident (AG-4 = 97%; AG-2-2 IIIB and IV = 99%; AG-2-1 = 85%; AG-9 = 99%; AG-6 and AG-7 = 75%). Two clusters of AG-4 isolates corresponded to HGI and HGII; AG-6 to HG-I and GV, and AG-9 to TX and TP subgroups based on previous DNA/DNA hybridization studies. Subgroups of AG-1 represented a distinct cluster based on neighbor joining tree, but had moderate support with bootstrap analysis (63%). Results from ITS1 rDNA sequence analysis in this study are consistent with the results of Kuninaga et al (1997). However, the combined analysis of the ITS and LSU region grouped AG-1-IA and AG-1-IC, but not AG-1-IB together. Also, the combined analysis of the ITS and LSU region grouped AG-4 HGI and AG-4 HGII, but not AG-4 HGIII. Therefore, the hypothesis that AG represents the most fundamental evolutionary units within *Thanatephorus* (anamorph = *R. solani*) was rejected.

The availability of a large ITS database allowed an expanded phylogenetic analysis of *R. solani*. The ITS region was very difficult to align and exhibited more homoplasy than the LSU region. Although this alignment generated many indels in the ITS region, most were concentrated in six highly variable regions. In our analyses only phylogenetically informative characters were used. We chose to delete gapped (ambiguous) and variable regions based on rigorous examination of the effect of removing these regions of data (data not shown). Although the highly variable ITS1 rDNA region was useful for identifying individuals, it may be inappropriate for phylogenetic analysis due to excessive nucleotide deletions, insertions and substitutions (Kuninaga et al 1997). Therefore, we conducted an analysis with combined ITS and LSU sequence data. This illustrates the importance of striking a balance between alignment, homoplasy and phylogenetically informative characters to achieve the desired level of taxonomic resolution.

Several ITS sequences available in GenBank were problematic and not included in our analysis. These sequences were very difficult to align and appeared as long branches in our phylogenetic tree, suggesting

the presence of many autapomorphies associated with sequencing errors. As part of this study, three isolates (1556, AH-1, and A68) previously sequenced by Kuninaga et al (1997) were re-sequenced (76Rs, ATCC 76126, and A68, respectively, in this study) to provide a measure of sequence quality. The sequences differed by two nucleotides, were easy to align and no long branches were evident in the subsequent analysis. With the accumulation of additional sequence data in the future, it will be very important to maintain high sequence quality to minimize misinterpretation of data by including previously sequenced isolates as an internal control.

The potential utility for unambiguous identification of isolates of *Ceratobasidium* and *Thanatephorus* is of practical significance to many mycologists and plant pathologists. Isolates 521 and Rh2815, originally described by Boysen et al (1996) as *R. solani* AG-4, were later identified as anamorphs of *Ceratobasidium* based on analysis of the rDNA ITS region (Kuninaga et al 1997, Boidin et al 1998, Salazar et al 1999). These isolates were placed in our data matrix and results suggest that they may belong to AG-A of *Ceratobasidium* (indicated in bold with a single asterisk * in FIG. 2). Also when isolate T62 of Johanson et al (1998) was placed in the data matrix, it grouped with AG-2–2 IIIB (indicated in bold with two asterisks ** in FIG. 2). In this study, we were unable to associate isolate T6 of Johanson et al (1998) with a specific AG (indicated in bold wih three asterisks *** in FIG. 2), which provided an indication of the limitations for sole use of rDNA-based data for identification. The accumulation of a larger and high quality rDNA sequence database should establish a foundation for the development of species concepts in *Rhizoctonia* and testing hypotheses related to geographic subdivision, host, and ecological specialization. For example, do ecologically interacting isolates share a common gene pool, host and/or geographic preference? The analysis of large data sets will present some challenges in the future, but DNA data coupled with additional characters should facilitate unambiguous identification of *Ceratobasidium* and *Thanatephorus*.

The majority of taxonomic studies have employed phenetic and distance based methods to infer phylogenetic relationships. Numerical methods through distance matrices group taxa based on overall similarity (a combination of synapomorphies, symplesiomorphies, and homoplasy) and not for their phylogenetic relationships. In a phenogram it is not possible to evaluate the contribution of each character in the formation of the groups. Therefore, there was a recognized need for a robust analysis well rooted in hypothesis testing to examine the phylogenetic relationships within *Ceratobasidium* and *Thanatephorus*. Despite the limitations of the bootstrap, they provide some indication of the internal support for the *Ceratobasidium* and *Thanatephorus* clades (Sanderson, 1989). Results from combined analysis of ITS and LSU sequence data suggest that AG-1, AG-4, AG-6, and AG-8 represent well-defined and genetically isolated groups, while AG-2 and AG-3 are of multiple origin (polyphyletic).

Although the taxonomic ranking of AG has been debated for decades, AG-4 was considered a distinct species by Kotila in 1929, prior to the development of the AG concept, and by other researchers since (Talbot 1970, Ogoshi 1987, Saksena and Vaartaja 1961, Anderson 1982). Information that has been accumulating in the past few years based on molecular data has provided additional support for AG-4 as a distinct species. However, we are not certain whether subgroups within AG-4 should be considered as species. We have taken a conservative approach and suggest that AG-4, but not its associated subgroups, represents a species that should be given formal taxonomic status.

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Capítulo III

PHYLOGENETIC UTILITY OF INDELS WITHIN rDNA AND BETA-TUBULIN SEQUENCES IN THE SPECIES COMPLEX *RHIZOCTONIA SOLANI*

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ABSTRACT

In gene sequences each position corresponds to a hypothesis of transformational or taxic homology depending if nucleotides vary or not. Indels are difficult to code, because they can be interpreted as an artifact from alignment or as phylogenetic information of mutational events. In this study, the effect of indels was examined on the stability of phylogenetic hypotheses. Parsimony analyses for a combined data set of a section of the beta-tubulin gene and rDNA sequences for *Rhizoctonia* anamorphs were performed with positions with indels excluded and included as ambiguous characters. To explore if they are a source of phylogenetic characters, single-site indels were treated as a new state, while indels longer than one nucleotide were coded as: 1) multistate for different sequence, 2) multistate for different length and 3) different characters for each distinct sequence. The best resolved cladograms and with higher support indexes were obtained when indels are included in the analyses. Indels recoded as different characters recovered historical information. Results indicated that the *R. solani* complex is not monophyletic. Also, six clades within *R. solani* (teleomorph = *Thanatephorus*) representing distinct anastomosis groups and five clades within binucleate *Rhizoctonia* (teleomorph = *Ceratobasidium*) are well supported in all analyses indicating they are natural groups. At the moment, we suggest that only clades with representatives of anastomosis groups 1, 4, 6 and 8 be recognized as species. Our results also suggest that indels in noncoding regions contain phylogenetic information.

INTRODUCTION

In DNA sequences the alignment corresponds to a set of hypotheses of the existence of a homology relationship for each nucleotide base position. Alignments of protein-coding genes do not represent any problem due to the presence of particular features such as reading frames or start and stop codons. In contrast, non-protein coding sequences are difficult to align. They lack these conventional features, and nucleotide base insertion and deletion (indels) events may occur when there are no selective constrains (Mindell, 1991). Alignment of this kind of sequences is a concern in phylogenetic analyses, especially when dealing with indels of different length. Once an alignment is accepted, nucleotide positions are hypothesized as homologous characters.

In morphology when a character is problematic non-conventional character coding approaches are adopted to code variation (Hawkins, 2000). In sequence data the identification of nucleotide positions is usually obvious, so there is no disagreement as to how we should code characters. However, indels of different length in a sequence alignment could make character state coding very problematic.

Epistemologically, indels can be interpreted as phylogenetic information of taxic or transformational homology (González, 1996), but also as an artifact. Methodologically, there has been little attention in generating a code strategy for indels that represent hypotheses of homology.

Irrespective of the alignment procedure used when dealing with problematic sequences, a common practice is to ignore indels as ambiguous characters or simply delete them from data sets (Olsen and Woese, 1993). The rationale for deleting sites with indels is that if they cannot be reliably aligned they are phylogenetically uninformative (Olsen, 1988; Olsen and Woese, 1993). Unfortunately, there are few objective criteria for deciding which sites with indels are ambiguous (Gatesy *et al.,* 1993).

In studies where indels are considered as putative insertion or deletion events, they are incorporated as a class of phylogenetic characters in the analyses (Lutzoni *et al*., 2000; Simmons and Ochoterena, 2000). However, they have not been widely accepted as phylogenetic markers because there are not sufficient empirical studies of the quality of gaps as characters (Kawakita *et al*., 2003).

In a previous study to investigate phylogenetic relationships within *Rhizoctonia solani* (teleomorph = *Thanatephorus*), we found that sequence alignment of ribosomal DNA (ITS and LSU) generated several gaps of different length (González *et al*., 2001). Choice among possible alignments, especially in the ITS region, was problematic. We took a conservative approach for parsimony analyses and chose the alignment with the highest penalty for introducing gaps and for the length of the gaps. The indels generated were coded as ambiguous characters. Under these parameters our conclusions were that *R. solani* was not monophyletic and that four unrelated clades represented well-defined genetically isolated groups (González *et al.,* 2001).

In this study, we tested the potential of gaps as phylogenetic characters by assessing their effect on the stability of phylogenetic hypothesis. We added more data and more taxa to our previous data matrix. Two new taxa had the longest sequence of the data matrix, therefore we tried alternative alignments. Positions with indels were excluded and included as ambiguous characters. To explore if they are a source of phylogenetic characters, single-site indels were treated as a new state, while indels longer than one nucleotide were coded as: 1) multistate for different sequence, 2) multistate for different length and 3) different

characters for each distinct sequence.

MATERIAL AND METHODS

Taxon sampling and molecular protocols. Forty-three isolates representing 28 anastomosis groups (AG) of binucleate *Rhizoctonia* spp., (teleomorph = *Ceratobasidium*) and multinucleate *Rhizoctonia solani* (teleomorph = *Thanatephorus*) were used in this study. Thirty-seven of these isolates were previously sequenced for the ITS and LSU of the rDNA (González *et al.*, 2001). The six new isolates were sequenced for the rDNA, additionally a section of the beta-tubulin gene of 368 nucleotides was sequenced for all forty-three isolates. Isolates come from USA, Japan, Canada, Australia and Scotland (Table 1).

DNA extraction, amplification and sequencing were performed as described in González *et al.*, (2001). Primers named "B36F" (5'-CACCCACTCCCTCGGTGGTG-3'), and "B12R" (5'-CATGAAGAAGTGAAGACGCGGGAA-3') were used to amplify and/or sequence the beta-tubulin region for most isolates. We designed two new primers "BTUB-1F" (5'-CACCCACTCWCTWGGTGGT-3') and "BTUB-380R" (5'-TACCCATGTTGACAGCRAG-3') for some isolates due difficulties to encountered obtaining PCR products.

Sequence Analyses. Sequences were aligned using the Clustal V program (Higgins *et al.*, 1992) within the Megalign software package (Lasergene, DNASTAR Inc). The aligned data set consisted of 43 OTUs and 2049 nucleotide positions from the ITS, LSU and beta-tubulin genes. A survey of primary homology assessment was performed. We did 12 alignments with combinations of penalties of 10, 20, 50 and 100 for introducing gaps and penalties of 5, 10, 20, 50 and 100 for the length of gaps. Some analyses were done coding indels of one nucleotide as an additional character state and recoding indels of two or more contiguous nucleotides. In these analyses we added the "dash" symbol in the "matrix format" in PAUP*, ver. 4.0b4 (Swofford, 2000), in order to consider indels of one nucleotide as a different character state. For indels of two or more nucleotides we first identified the positions in the matrix that contained the gapped sequences. Then, we removed all gaps from these positions, and finally recoded indels to represent variation. Coding large indels was problematic when some taxa have one combination of nucleotides in the sequence, others have a different combination, and the remaining taxa lack the sequence altogether. Three approaches were used to describe variation of indels of two or more positions. First, a single multistate treatment as described by Lutzoni *et al.* (2000). Second, a multistate code to identify only a specific length of the indel; and third a different character for describing a specific nucleotide sequence (Fig. 1). With these codes we are interpreting the origin of indels of two or more positions as a single insertion-deletion event. Indels that presented one or more unidentified nucleotide (N) in its sequence (no matter the length of the sequence) were coded as ambiguous character.

Distance Analysis. Sequence distances and standard distances among taxa were measured in PAUP*, ver. 4.0b4 (Swofford, 2000). Sequence distances for the combined data set with indels included as ambiguous characters and excluded were obtained by maximum-likelihood (ML) analysis, with the model of DNA substitution chosen with MODELTEST (Posada and Crandall, 1998). Standard distances were acquired for the three data sets where indels were recoded.

Phylogenetic Analyses. All analyses were performed with individual and combined

data sets using the maximum parsimony criterion in PAUP*, ver. 4.0b4 (Swofford, 2000). We used heuristic searches with 500 random addition replicates and TBR branch swapping after exclusion of uninformative characters. Branch support was assessed by Bremer support index, and bootstrap analysis based on 500 heuristic replicates for most analyses. Despite the limitations of the bootstrap, it provides some indication of internal support (Sanderson, 1989). For the LSU data set only 10 replicates of bootstrap were possible, for the LSU+TUB data only 98. The tubulin data set alone was not analyzed with bootstrap due the high number of trees generated in the first replicate. Data decisiveness (DD) (Kitching *et al.,* 1998) was calculated as a measure of information content in individual and combined data sets. The orientation of the tree was performed with sixteen AGs of binucleate *Rhizoctonia* spp., (teleomorph = *Ceratobasidium*) used as outgroup.

Topological congruence was evaluated among alternative taxonomic hypotheses produced with the combined three gene partitions and indels excluded and recoded, and then testing these against the most-parsimonious trees obtained when all indels are coded as ambiguous characters. We used Templeton nonparametric and Kishino-Hasegawa parametric tests (P = 0.05 as level of significance) as implemented in PAUP*. For this purpose we utilized one randomly chosen cladogram from the analysis with all indels included as ambiguous characters and compared it with the cladograms obtained in the analyses with indels excluded and recoded.

RESULTS

Sequence Analyses. The tubulin gene and the LSU had almost no alignment problems. Gapped sites were frequent in the ITS region. Among the twelve alignments generated, we chose those that did not modify the 5' and 3' ends of the 5.8S of the rDNA. From these, the alignment with a gap penalty of 20 and a gap length penalty of 10 was selected. We did few adjustments by visual examination for analyses. This alignment generated 74 indels of one nucleotide and 34 indels of two or more contiguous nucleotides (Table 2).

After alignment, the sequence had 2049 characters. From these, 1398 (68%) were constant, 196 (10%) were variable but parsimony uninformative, and 455 (22%) were parsimony informative. The removal of indels reduced informative characters to only 296 (14%). Recoding indels as multistate characters produced 2083 characters, 368 (18%) of these were informative while using a multistate code to identify an indel of specific length originated 367 informative characters. Indels coded as different characters for describing a specific nucleotide sequence resulted in 2301 characters. From these 476 (21%) were informative. The frequency distribution of 100,000 random tree lengths for each region separately or combined was left-skewed (g1 statistic) indicating that data has phylogenetic signal (Hillis and Huelsenbeck, 1992). The ITS data set alone or combined with other gene partitions showed the lowest g1 values. The data decisiveness score (DD) was higher for the tubulin sequences than any other gene partition. On the other, hand the lowest DD score corresponded to the three gene partitions combined and all indels excluded reflecting more internal conflicts in this data matrix (Table 3).

Nucleotide composition for the combined data matrix and all characters included, varied little among taxa (A = 0.256, C = 0.222, G = 0.247, T = 0.273), with an average A + T composition of 53%. The tubulin gene region was the gene partition that had the largest nucleotide composition variation (A = 0.058, C = 0.481, G = 0.178, T = 0.281), with an

average A + T of 34%. A chi-square test of homogeneity of base frequencies across taxa for the combined data matrix and all characters included gave 22.993 (df = 126, P = 1.0000); hence nucleotide composition was homogeneous. Homogeneity was also seen for each gene partitions and for the combined data matrix with indels excluded.

Distance Analysis. MODELTEST chose the modification of the GTR + I + G (where GTR is general time reversible, I is the proportion of invariable sites, and G is the shape parameter of the gamma distribution) model of DNA sequence evolution as most appropriate for our data. The model parameters used in the ML distance analysis for the combined three gene partitions and all indels included as ambiguous characters were as follows: base frequencies = 0.2502, 0.2198, and 0.2407; NST (number of substitution types) = 6; Revmat (rate matrix) = 0.7999, 2.0641, 1.6424, 0.9975, and 3.6643; rates = gamma; alpha parameter of the gamma distribution = 0.4495; and proportion of invariant sites (Pinvar) = 0.5192. The model parameters for the three gene partitions and all indels excluded were: base frequencies = equal; NST = 6; Revmat = 0.5911, 1.9432, 1.2817, 0.9506, and 4.0895; rates = gamma; alpha parameter of the gamma distribution = 0.5742; and proportion of invariant sites (Pinvar) = 0.6204.

Distance values under the GTR + I + G model for the combined three gene partitions and all indels included as ambiguous characters varied from 0.0074 between the isolates 7Rs (AG4-HGII) and 30Rs (AG4-HGII), to 1.7659 between C-610 (AGD) and 1Rs (AG1-IA). Distance values when indels are excluded ranged from 0.0043 between the isolates 7Rs (AG4-HGII) and 30Rs (AG4-HGII) to 0.1954 between C-610 (AGD) and BN37 (CAG5).

Mean character differences adjusted for missing data when indels were coded as multistate characters varied from 0.0112 between isolates 7Rs (AG4-HGII) and 30Rs (AG4-HGII), to 0.5650 between C-610 (AGD) and 1Rs (AG1-IA). When indels were coded to identify a specific length, distance varied almost similarly as the multistate code. When indels are coded using a different character for describing the combination of nucleotide of the sequence, the distances were 0.0087 between isolates 7Rs (AG4-HGII) and 30Rs (AG4-HGII), and 0.4838 between C-610 (AGD) and (ZG9)91614 (AG10).

Phylogenetic Analyses. A comparison of general features of the most parsimonious trees (MPTs) found in all seventeen analyses performed is summarized in table 3. Different resolution levels of phylogenetic relationships were reconstructed in our analyses with the three data sets taken individually and when indels were included as ambiguous characters or excluded (trees not shown). The strict consensus obtained with the LSU sequences had the poorest resolution due to competing topologies. Even the consensus of 113,776 trees found with the 368 nucleotides sequenced from the tubulin gene had better resolution in basal branches than the LSU tree. In contrast, the strict consensus of the ITS sequences was well resolved and supported in terminal branches but less resolved in basal branches. In the analyses of each gene partition there was a better resolution when the nucleotide positions with indels were included. Similar results were obtained for the combined analysis of LSU and tubulin. In contrast, other combinations of two gene partitions (ITS+LSU and ITS+TUB) had better resolution when these positions were excluded. The clades obtained with bootstrap values above 50% in at least five of the ten analyses for each gene partition and in combination when positions with gaps were included or excluded are described in table 4. For the tubulin data set alone bootstrap analysis could not be accomplished because of the high number of trees generated in the first replica. Six clades of isolates of the same AG of *R. solani* (AG1, AG4, AG6, AG8, AG10 and AGBI), one clade of subgroup AG2-2 and five clades of *Ceratobasidium* (AGA,AGBo; AGD,CAG1; AGF,CAG5;

AGBb,AGBa,AGQ; and AGG,AGL,AGO) are always recovered with high support values. In clades containing isolates of AG1, AG4, AG8, AGBb-AGBa-AGQ, and AGG-AGL-AGO internal relationships of AG or subgroups varied.

From all seventeen analyses (individual or combined data sets with indels included as ambiguous characters, excluded or recoded), the best resolution was obtained when the three data partitions were combined, and gapped positions were included and coded as ambiguous characters (6 trees) or when indels were recoded. From these last analyses, the best resolution was when indels were coded using a different character for describing the combination of nucleotide of the sequence (9 trees). In contrast, the analysis with three gene partitions but gapped positions excluded had the poorest resolution (177 trees) (Fig.2).

In each consensus tree obtained when the three gene partitions are combined, six isolates from *Ceratobasidium* spp., anastomosis group CAG3, AGF, CAG5, CAG6, CAG4 and CAG7, are mixed and dispersed across several small clades of *R. solani*, indicating these groups are not monophyletic (Fig. 2). In all analyses, trees always show one clade with eleven isolates of *Ceratobasidium* (AGA, AGBo, AGG, AGL, AGO, AGH, AGD, CAG1, AGQ, AGBa and AGBb) (Fig. 2), which is sister to a larger clade containing all 26 isolates of *R. solani* (AG1 to 11 and BI) and CAG3, CAG4, CAG5, CAG6, CAG7, and AGF. Internally, the clade containing only isolates of *Ceratobasidium* consistently accommodates five clades [(AGA,AGBo); ((AGD,CAG1)(AGQ(AGBb,AGBa))); (AGH((AGD,CAG1)(AGQ(AGBb,AGBa)))); (AGBb,AGBa); and (AGG((AGL,AGO))]. The larger clade contains eight well-supported small clades (table 5). Six of these clades include only isolates of a same anastomosis group of *R. solani* (AG1, AG4, AG6, AG8, AG10 and AGBI), one included one subgroup of *R. solani* (AG2-2) and other two binucleate *Ceratobasidium* isolates AGF and CAG5. These eight clades are also present in most analyses with one or two gene partition (table 4). This indicates that there are phylogenetically informative regions in the three gene partitions used and they are relatively robust to different strategies for coding the indels. In contrast with the constancy of the clades formed, their relationships are not stable. Most variation among trees was due to local rearrangement of taxa (Fig. 2). It is clear that the placement of clades is sensitive to gene partition and the strategy used for coding indels. Results of Kishino-Hasegawa and Templeton tests to evaluate alternative taxonomic hypotheses indicated that trees obtained in the analysis where indels are included as ambiguous characters were significantly less parsimonious than trees obtained in analyses where indels were excluded or recoded. The most significant results with both tests were obtained between trees where indels were excluded versus indels coded as ambiguous characters (P value = 0.0010 and 0.0020 respectively).

DISCUSSION

In any kind of data, characters are the units of language through which the ideas of homology, relationships, diagnosis and identity are communicated (Forey and Kitching, 2000). Thus, any error in sequence alignment will lead to potential mistaken hypotheses of homology and consequently, to different systematic outcomes. Molecular phylogenies rely predominantly or exclusively on substitutions. However, from a molecular point of view, gaps also originate from particular biological events such as mutation (insertion or deletion) and thus they may contain as much historical information as observed in nucleotide changes (Giribert and Wheeler, 1999). It has also been suggested that indels that extend over a

number of residues in sequence data may have a sufficiently low probability of occurring that they could be used to identify monophyletic groups (Lloyd and Calder, 1991, Kawakita *et al.*, 2003).

In this study, most gapped regions are restricted to certain nucleotide positions, which indicate they may be linked biologically. Therefore, it is better to use a code that represents the variation for expressing such connection than treat them as ambiguous characters ("?") or to exclude them from the analyses. It is true that recoding indels with a code such as multistates many times requires a high number of character states, especially when trying to recode large regions with extreme differences in length. Coding this kind of regions with a large number of character states raises the question if they should be included. We performed analyses including and excluding four large regions coded this way. Resolution of the consensus tree obtained changed but grouping did not change substantially.

Our results may appear unsatisfactory when only strongly supported and non-contradictory branches are considered. Nevertheless we found that support for most clades increased when the three gene partitions were combined, with the exception of AG8 where support was higher in individual analyses. Also, two large clades can be recognized within *Rhizoctonia* anamorphs. One clade, with only binucleate *Rhizoctonia* spp., (teleomorph = *Ceratobasidium*) is sister to a clade of all 26 isolates of multinucleate *Rhizoctonia solani* (teleomorph = *Thanatephorus*) and 6 anastomosis groups of *Ceratobasidium*. Both clades contain several well-supported smaller clades. However, the relationships among these smaller clades are not resolved in different analyses or they are not sufficiently supported. In spite of that, the phylogenetic analyses of *R. solani* (teleomorph = *Thanatephorus*) and binucleate *Rhizoctonia* spp. (teleomorph = *Ceratobasidium*), with each gene partition and in combination and with indels included as ambiguous characters, excluded or recoded are congruent among them in many parts. For instance, six clades of isolates of the same AG of *R. solani* (AG1, AG4, AG6, AG8, AG10 and AGBI), one subgroup (AG2-2) and five clades of binucleate *Rhizoctonia* (AGA-AGBo; AGD,CAG1; AGF,CAG5; AGBb,AGBa,AGQ; and AGG,AGL,AGO) were always recovered with high support values.

The hypothesis that *Thanatephorus* is paraphyletic with respect to *Ceratobasidium* (González *et al.,* 2001) is again supported by these analyses. There is a high bootstrap and Bremer support indexes for a clade containing all 26 isolates of *R. solani* (AG1 to 11 and BI) and 6 binucleate (CAG3, CAG4, CAG5, CAG6, CAG7, and AGF). These results suggest that at least some isolates of *Ceratobasidium* may be more closely related with other isolates from *Thanatephorus*.

Vegetative characteristics such as mycelia coloration, hyphal diameter, number of nuclei, length of cells, shape and size of monilioid cells, and sclerotial size have generally been used in the characterization of *Rhizoctonia* spp. However, these characteristics vary considerably with temperature, light and composition of the medium (Andersen, 1990; Stalpers and Andersen, 1996). It has been observed that many isolates of *Thanatephorus* and *Ceratobasidium* often have a similar appearance when grown on nutrient medium, and hyphal fusion among isolates can occur (Kotila 1929, Parmeter *et al.*, 1967, Burpee *et al.*, 1980, Ogoshi 1987). Besides, nuclear number in vegetative cells is rarely absolute. For example, *Thanatephorus* (anamorph = *R. solani*) is considered to be multinucleate (Parmeter and Whitney, 1970), but nuclear condition is variable, cells of a strain may display a large variation in number (e.g., 2-18) (Tu *et al.*, 1977). Also, *Ceratobasidium*

(anamorph = *Rhizoctonia* spp.) is generally binucleate (Parmeter *et al*., 1967, Burpee *et al*., 1980), but *C. koleroga* has been reported to be multinucleate (Stalpers and Andersen, 1996). This evidence suggests that certain isolates of *Ceratobasidium* and *Thanatephorus* are closely related. However, more testing with additional isolates of *Ceratobasidium* is required to substantiate the relationships of the anastomosis groups CAG3, CAG4, CAG5, CAG6, CAG7, and AGF with *Thanatephorus*.

The results of this study provide evidence for the monophyly of AG1, AG4, AG6, AG8, AG10 and AGBI of *R. solani*, and the polyphyletic origin of AG2, also indicate that coding indels as different characters for each distinct sequence best recover the phylogenetic information. Bootstrap and Bremer support on basal branches are higher when this scoring scheme was used. Data decisiveness score is also high indicating this data set has less internal conflicts than when indels are excluded or included as ambiguous characters. The lowest DD score and retention index found was for the three gene partitions combined and indels excluded.

The hypothesis of relationships obtained when indels are coded as different characters placed CAG3 as sister group of AG1 (Fig 2-E). This clade is closer to the root with respect to the rest of *R. solani* (teleomorph = *Thanatephorus*) and one clade formed by *Ceratobasidium* isolates. AG2-2 resulted sister taxa of AG4. The clades represent at least 17 species within *Thanatephorus* and *Ceratobasidium*. Most of these clades are congruent with current grouping based on anastomosis. The lack of stability of subgroups within anastomosis groups in the different analyses performed led to conclude that smaller clades within *R. solani* (at subgroup level) represent genetic variation within anastomosis groups and not taxonomic species.

Our data matrix is sensitive to different alignment parameters. This is a matter of concern in this study and many others with sequences of different length. Therefore, it is important to investigate the phylogenetic content of regions containing multiple indels. As with a complex morphological feature, there may be multiple possible ways of coding indels, the correct approach is still unknown. However, including them with an alternative coding scheme allows the opportunity to evaluate them as hypotheses which are subject to the cladistic test of congruence in a parsimony analysis.

Templeton's (1983) nonparametric test has been used to evaluate tree topologies by constraining phylogenetic searches to seek trees consistent with alternative taxonomic hypotheses and then testing these against the most-parsimonious trees. In this study we used this test to compare the most parsimonious trees obtained in the analyses where indels were excluded, considered as ambiguous characters or recoded. Under this perspective, trees obtained in the analysis where indels were treated as ambiguous characters were significantly less parsimonious than trees obtained in analyses where indels were excluded or recoded.

There is still an ongoing debate on how to test alternative phylogenetic hypothesis. Several tests have been proposed to compare two trees for either their parsimony or maximum likelihood scores. Templeton's test is a Wilcoxon signed-rank test that compares the differences at each site in the number of substitutions required for each tree in a parsimony framework. It has been widely used to test taxonomic congruence (e.g. Cunningham, 1997; Hardig et. al., 2000; Magombo, 2003), but as with many other tests, it has been criticized. This test does not make assumptions about the distribution of substitution differences at each site therefore it does not take into consideration that differences may not be symmetrical neither normally distributed. If we carry out the test on

data with substitution differences at each site non-normally distributed, it may give an incorrect answer to the significance of the difference between the trees being compared (Felsenstein, 2004). Other tests have been proposed under a parsimony framework such as the Kishino-Hasegawa test. This test, in contrast with Templeton's is parametric. The significance is judged by comparing the length difference to the variance in the tree length difference over all sites. With our data both tests behave similarly.

The results of this study did not fully solve the problem of relationships nor classification within *Rhizoctonia* spp. The fact that clades were not stable revealed that more taxa and, perhaps another marker that reinforces support on basal branches, should be added to obtain a more reliable classification (Graybeal, 1998; Poe, 1998). Another marker might also help to define more precisely how many species are there in these groups based on their phylogenetic relationships. By the moment we cannot discard the hypothesis that each anastomosis group within *R. solani* represents a genetically isolated group. However, at this time we will take a conservative approach and recommend that only clades formed by isolates of AG1, AG4, AG6 and AG8 of *R. solani* (teleomorph = *Thanatephorus*) be recognized as different species (Fig 2-E). The available scientific name for the clade including isolates of AG1 is *Thanatephorus sasaki* (Shirai) Tu & Kimbrough. For the clade with isolates of AG4 the available name is *T. praticola* (Kotila) Flentje. For the clade with isolates of AG6 and the clade containing isolates from AG8 there is no existing name (in preparation).

The precise strategy for coding indels for phylogenetic studies still remains to be established, but judging for the results obtained in this study, it seems that coding them as different characters for each distinct sequence recovered historical information. Therefore, we recommend that cladistic analyses with gene sequences of different length should have an evaluation of the effects of including indels on the stability of phylogenetic hypothesis. Considering our results we conclude that indels can be good characters, at lower taxonomic levels, but we also recognize that gaps, like all classes of phylogenetic characters, are not devoid of homoplasy.

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Table 1. Anastomosis (AG/CAG) and subgroup designation, origin and source of isolates of *Rhizoctonia* species with *Ceratobasidum* and *Thanatephorus* teleomorphs used for sequence analysis of the internal transcribed spacer (ITS1 and 2), adjacent 28S region and a section of the beta-tubulin gene and their GenBank accession number.

Anastomosis group/subgroup	Isolate	Origin (Source)[1/]	GenBank accession number	
			rDNA (ITS and 28S)	beta-tubulin
Thanatephorus (*R. solani*)				
AG-1-IA	1Rs (ATCC 66159)	Soybean, US (11)	AF354060	
AG-1-IB	36Rs (ATCC 66150)	Turfgrass, US (2)	AF354059	
AG-1-IC	3Rs (ATCC 44661)	Pine, Canada (1)	AF354058	
AG-2-1	8Rs (ATCC 44658)	Soil, Australia (1)	AF354063	
AG-2-2	9Rs (ATCC 44659)			
AG-2-2 IV	16Rs	Sugar beet, Japan (10)	AF354117	
AG-3	4Rs (ATCC 14006)	Potato, US (9)	AF354064	
AG-4 HGI	AH-1 (ATCC 76126)	Peanut, Japan (7)	AB000012, AF354118	
AG-4 HGII	7Rs (ATCC 44662)	Alfalfa, US (1)	AF354074	
	18Rs	Sugar beet, Japan (10)	AF354072	
	30Rs (ATCC 48803)	Unknown, Canada (3)	AF354073	
AG-4 HGIII	6Rs (ATCC 42127)	Conifer, US (3)	AF354077	
	45Rs (ATCC 10177)	Sugar beet, US (6)	AF354076	
AG-5	10Rs	Soybean, Japan (10)	AF354078	
AG-6 HG-I	72Rs	Soil, Japan (10)	AF354061	
AG-6 GV	74Rs	Soil, Japan (10)	AF354062	
AG-7	76Rs	Soil, Japan (4)	AF354096	
AG-8	(ZG1-2)SA50	Oats, Australia (5)	AF354067	
	(ZG1-3)SA1512	Barley, Australia (12)	AF354068	
	(ZG1-5)92547	Barley, Australia (12)		
AG-9	116Rs	Potato, US (4)	AF354065	

AG-10	W45b3	Wheat, US (10)	AF354111
	(ZG9)91614	Barley, Australia (8)	AF354071
AG-11	(ZG-3)R1013	Lupine, Australia (13)	AF354079
AG-BI	22Rs	Soil, Japan (10)	AF354070
	TE 2-4	Soil, Japan (10)	AB000044,
Ceratobasidium (binucleate *Rhizoctonia* spp.)			
AG-A	C-662	Soil, Japan (10)	AF354092
AG-Ba	C-460	Rice, Japan (10)	AF354088
AG-Bb	C-455	Rice, Japan (10)	AF354087
AG-Bo	SIR-2	Sweetpotato, Japan (10)	AF354091
AG-D	C-610	Unknown, Japan (10)	AF354090
AG-F	SIR-1	Sweetpotato, Japan (10)	AF354085
AG-G			
AG-H	STC-9	Soil, Japan (10)	AF354089
AG-L	FK02-1	Soil, Japan (10)	AF354093
AG-O	FK06-2	Soil, Japan (10)	AF354094
AG-Q	C-620	Soil, Japan (10)	AF354095
CAG-1	BN1	Turfgrass, US (2)	AF354086
CAG-3	BN31	Peanut, US (2)	AF354080
CAG-4	BN38	Soybean, US (2)	AF354081
CAG-5	BN37	Cucumber, US (2)	AF354082
CAG-6	BN74 (ATCC 13247)	*Erigeron*, US (2)	AF354083
CAG-7	BN22 (FL FTCC585)	*Pittosporum*, US (2)	AF354084

[1/] Isolates provided by; 1=N. Anderson; 2=L. Burpee; 3=E. Butler; 4=D. Carling; 5=A. Dube; 6=J. Kotila; 7=S. Kuninaga; 8=G. MacNish; 9= G. Papavizas; 10=A. Ogoshi; 11=N. O'Neill; 12=S. Neate; and 13=M. Sweetingham

Table 2. Number of characters in the sequence before and after alignment.

Gene partition	Before		After	Indels of one nucleotide	Indels of two or more nucleotides
	shortest	longest			
ITS	571	654	730	21	23
LSU	920	936	954	53	10
TUBULIN	313	365	365		1

Table 3. Attributes of three character sets (ITS, LSU, Tubulin) and combinations of them, when indels are included as ambiguous characters (?), when are excluded, and when are re-coded (A = multistate for different sequence; B = multistate for different length; C = different characters for each distinct sequence). MPT = most parsimonious trees; CI = consistency index; RI = retention index; DD = data decisiveness.

Gene partition	Inform.	# of MPT	Length of MPT	CI	RI	DD
ITS						
indels as ?	289	7	1255	0.4191	0.6240	0.5691
excluded	144	29	491	0.4603	0.6764	0.6394
LSU						
indels as ?	93	4726	266	0.4737	0.6585	0.6153
excluded	79	11323	221	0.4842	0.6535	0.6135
TUB						
indels as ?	73	>113776	262	0.4084	0.7002	0.6509
excluded	73	>113776	262	0.4084	0.7002	0.6509
ITS+LSU						
indels as ?	382	8	1568	0.4158	0.6100	0.5544
excluded	223	86	754	0.4416	0.6333	0.5916
ITS+TUB						
indels as ?	362	78	1706	0.3710	0.5631	0.4976
excluded	217	114	910	0.3659	0.5681	0.5110
LSU+TUB						
indels as ?	166	28	641	0.3635	0.5599	0.4950
excluded	152	2660	580	0.3690	0.5674	0.5054
ITS+LSU+TUB						
indels as ?	455	6	2028	0.3743	0.5572	0.4924
excluded	296	177	1190	0.3697	0.5495	0.4918
A	368	24	1674	0.4337	0.5734	0.5187
B	367	15	1590	0.3975	0.5653	0.5097
C	476	9	1739	0.3755	0.5748	0.5184

Table 4. Bootstrap percentajes for subset of the clades suppor by bootstrap values above 50% in at least five analyses for each gene partition and in combination when positions whit indels are included as ambiguous characters ("?"), or excluded.

CLADE	Indels included as "?"					Indels excluded				
	ITS	LSU	ITS+ LSU	ITS+ TUB	LSU+ TUB	ITS	LSU	ITS+ LSU	ITS+ TUB	LSU+ TUB
AGA,AGBo	100	100	100	100	100	100	100	100	100	100
AGG,AGL,AGO	85	93	99	77	89	97	90	99	81	55
AGD,CAG1	100	70	100	100	98	100	80	100	100	100
AGBb,AGBa,AGQ	86		73	72		59			58	
AG1-IA,AG1-IB,AG1-IC	92	85	99			73		95		
AG2-2,AG2-2	100		100	100		100		100	87	
AG4-HGIII,AG4-HGIII,AG4-HG1,AG4-HGII,AG4-HGII,AG4-HGII	75		89	76		77		87		
AG6-HGI,AG6-HGV	92		94	99	87	56		86	91	88
AGBI,AGBI	100	100	100	100		100	100	100	100	
AG8(ZG1-2),AG8(ZG1-3), AG8(ZG1-5)	100		100	82		91		97		
AG10,AG10	100		100	99		100		100	78	
AGF,CAG5	100		100	100		100		100	97	

Table 5. Bootstrap percentajes for subset of the clades suppor by bootstrap values above 50% in at least four analyses with three gene partitions and indels recoded.

CLADES	as ambiguous characters	excluded	multistate	multistate for different length	different character
AGA,AGBo,AGH,AGD,CAG1,AGBa,AGBb, AGQ, AGG,AGL,AGO/All *R. solani* (AG1 to 11 and BI),CAG3,CAG4,CAG5,CAG6,CAG7,AGF		100	100	100	100
AGA,AGBo	100	100	100	100	100
AGG/AGL,AGO	96	92	97	95	98
AGH/AGD,CAG1,AGQ,AGBa,AGBb		89	92	87	94
AGD,CAG1/AGQ,AGBa,AGBb		68	64	63	57
AGBb,AGBa		50	74	73	74
AG1-IA,AG1-IB,AG1-IC	83	52	86	85	94
AG10,AG10	99	85	99	99	100
AG2-2,AG2-2	100	91	100	100	100
AG6-HGI,AG6-HGV	99	95	99	98	100
AGBI,AGBI	100	100	100	100	100
AGF,CAG5	100	99	100	100	100
AG4-HG1/AG4-HGIII,AG4-HGIII,AG4-HGII,AG4-HGII,AG4-HGII	83	79	93	93	92
AG8(ZG1-3)/AG8(ZG1-2),AG8(ZG1-5)	91		72	61	91

A

			as ambiguous characters
1	ACGCC------TTTTAACATT----TA	1	ACGCC??????TTTTAACATT????TA
2	ACGCCCATTCATTT----G------TA	2	ACGCCCATTCATTT????G??????TA
3	ACGCCCATTCATTT----G------TA	3	ACGCCCATTCATTT????G??????TA
4	ACGCTCCA----TTAA----TTTGGTA	4	ACGCTCCA????TTAA????TTTGGTA
5	AGGCCCATTCATTT----G------TA	5	AGGCCCATTCATTT????G??????TA
6	AGGCTCCA----TTA---GTT-TGGTA	6	AGGCTCCA????TTA???GTT?TGGTA
7	ACGCCCTTCAATTTAGCTGTTCTGGTA	7	ACGCCCTTCAATTTAGCTGTTCTGGTA
8	ACGCCCTTCAATTTAACTGTTCTGGTA	8	ACGCCCTTCAATTTAACTGTTCTGGTA
9	ACGCTCCA----TTAA---TT-TGGTA	9	ACGCTCCA????TTAA???TT?TGGTA

B

			excluded
1	ACGCC------TTTTAACATT----TA	1	ACTA
2	ACGCCCATTCATTT----G------TA	2	ACTA
3	ACGCCCATTCATTT----G------TA	3	ACTA
4	ACGCTCCA----TTAA----TTTGGTA	4	ACTA
5	AGGCCCATTCATTT----G------TA	5	AGTA
6	AGGCTCCA----TTA---GTT-TGGTA	6	AGTA
7	ACGCCCTTCAATTTAGCTGTTCTGGTA	7	ACTA
8	ACGCCCTTCAATTTAACTGTTCTGGTA	8	ACTA
9	ACGCTCCA----TTAA---TT-TGGTA	9	ACTA

C

			multistate code
1	ACGCC------TTTTAACATT----TA	GCCTTTTAACATT	0
2	ACGCCCATTCATTT----G------TA	GCCCATTCATTTG	1
3	ACGCCCATTCATTT----G------TA	GCCCATTCATTTG	1
4	ACGCTCCA----TTAA----TTTGGTA	GCTCCATTAATTTGG	2
5	AGGCCCATTCATTT----G------TA	GCCCATTCATTTG	3
6	AGGCTCCA----TTA---GTT-TGGTA	GCTCCATTAGTTTGG	4
7	ACGCCCTTCAATTTAGCTGTTCTGGTA	GCCCTTCAATTTAGCTGTTCTGG	5
8	ACGCCCTTCAATTTAACTGTTCTGGTA	GCCCTTCAATTTAACTGTTCTGG	6
9	ACGCTCCA----TTAA---TT-TGGTA	GCTCCATTAATTTGG	2

D

multistate code for different length

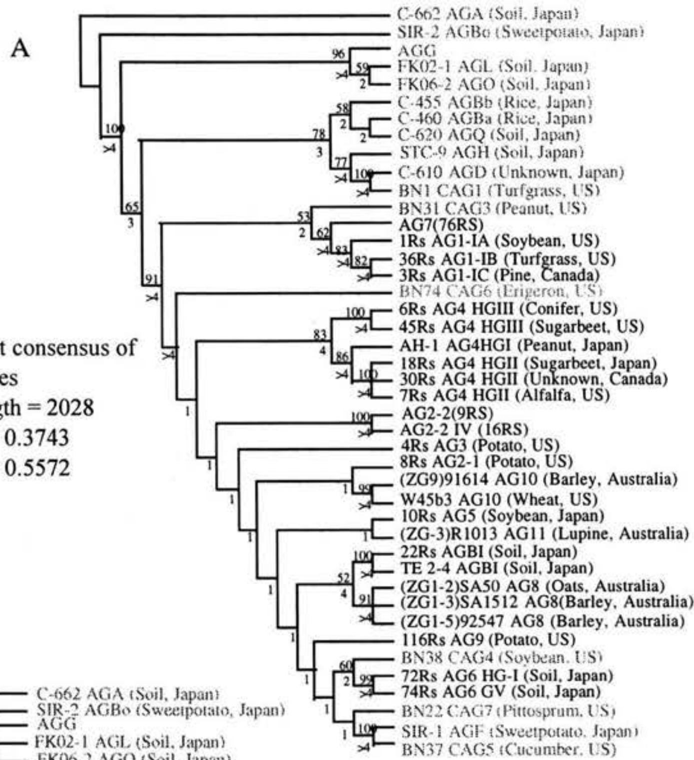
```
1  ACGCC------TTTTAACATT----TA      GCCTTTTAACATT                 1
2  ACGCCCATTCATTT----G------TA      GCCCATTCATTTG                 1
3  ACGCCCATTCATTT----G------TA      GCCCATTCATTTG                 1
4  ACGCTCCA----TTAA----TTTGGTA      GCTCCATTAATTTGG               2
5  AGGCCCATTCATTT----G------TA ---> GCCCATTCATTTG           --->  1
6  AGGCTCCA----TTA---GTT-TGGTA      GCTCCATTAGTTTGG               2
7  ACGCCCTTCAATTTAGCTGTTCTGGTA      GCCCTTCAATTTAGCTGTTCTGG       3
8  ACGCCCTTCAATTTAACTGTTCTGGTA      GCCCTTCAATTTAACTGTTCTGG       3
9  ACGCTCCA----TTAA---TT-TGGTA      GCTCCATTAATTTGG               2
```

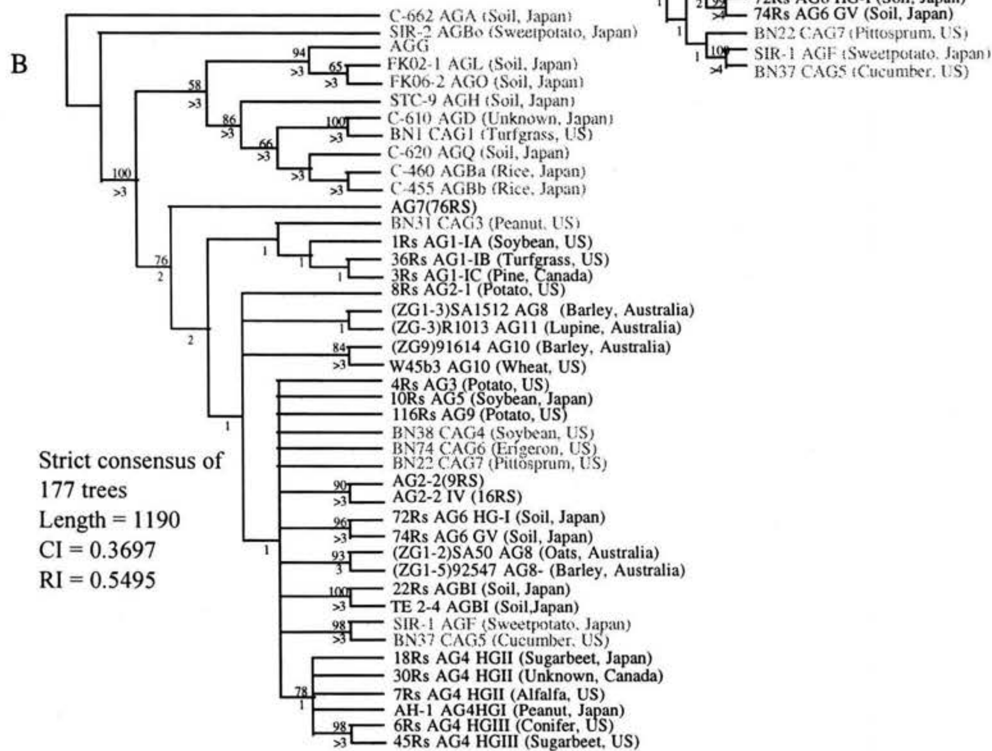
E

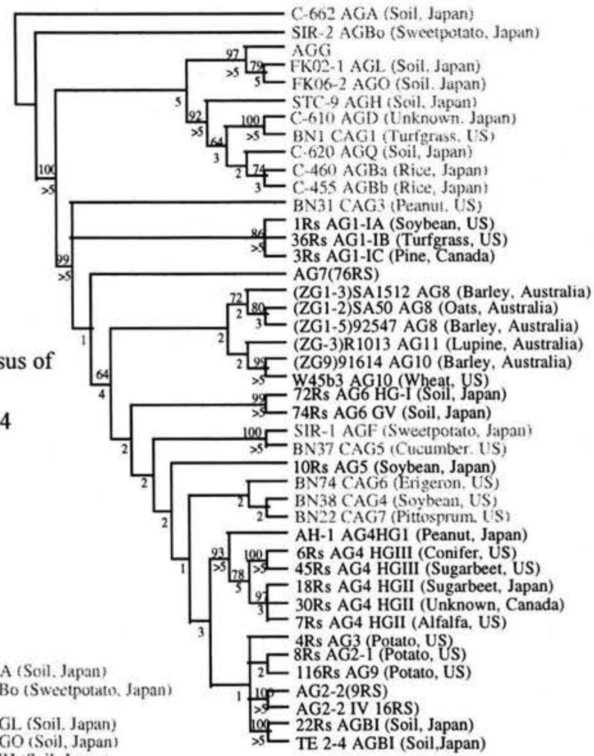
different characters

```
1  ACGCC------TTTTAACATT----TA      GCCTTTTAACATT                 1000
2  ACGCCCATTCATTT----G------TA      GCCCATTCATTTG                 0100
3  ACGCCCATTCATTT----G------TA      GCCCATTCATTTG                 0100
4  ACGCTCCA----TTAA----TTTGGTA      GCTCCATTAATTTGG               0010
5  AGGCCCATTCATTT----G------TA ---> GCCCATTCATTTG           --->  0100
6  AGGCTCCA----TTA---GTT-TGGTA      GCTCCATTAGTTTGG               0020
7  ACGCCCTTCAATTTAGCTGTTCTGGTA      GCCCTTCAATTTAGCTGTTCTGG       0001
8  ACGCCCTTCAATTTAACTGTTCTGGTA      GCCCTTCAATTTAACTGTTCTGG       0002
9  ACGCTCCA----TTAA---TT-TGGTA      GCTCCATTAATTTGG               0010
```

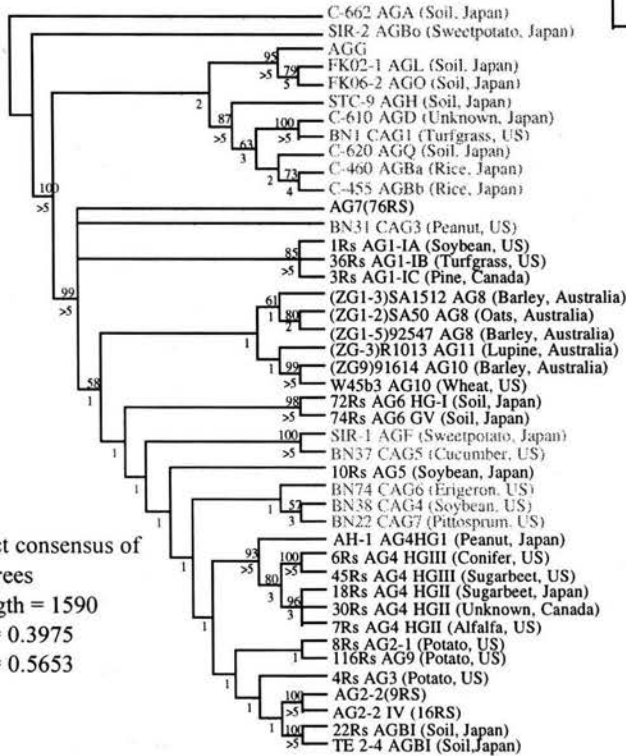
FIG 1. Different codes used for indels of two or more contiguous nucleotides. First, the positions in the matrix that contain the gaped sequences were identified. Second, all gaps from these positions were removed. Finally indels were recoded to represent variation. A) as ambiguous characters ("?"); B) excluded; C) as multistate for different sequence; D) as multistate for different length; E) as different characters for each distinct sequence


A
C-662 AGA (Soil, Japan)
SIR-2 AGBo (Sweetpotato, Japan)
AGG
FK02-1 AGL (Soil, Japan)
FK06-2 AGO (Soil, Japan)
C-455 AGBb (Rice, Japan)
C-460 AGBa (Rice, Japan)
C-620 AGQ (Soil, Japan)
STC-9 AGH (Soil, Japan)
C-610 AGD (Unknown, Japan)
BN1 CAG1 (Turfgrass, US)
BN31 CAG3 (Peanut, US)
AG7(76RS)
1Rs AG1-IA (Soybean, US)
36Rs AG1-IB (Turfgrass, US)
3Rs AG1-IC (Pine, Canada)
BN74 CAG6 (Erigeron, US)
6Rs AG4 HGIII (Conifer, US)
45Rs AG4 HGIII (Sugarbeet, US)
AH-1 AG4HGI (Peanut, Japan)
18Rs AG4 HGII (Sugarbeet, Japan)
30Rs AG4 HGII (Unknown, Canada)
7Rs AG4 HGII (Alfalfa, US)
AG2-2(9RS)
AG2-2 IV (16RS)
4Rs AG3 (Potato, US)
8Rs AG2-1 (Potato, US)
(ZG9)91614 AG10 (Barley, Australia)
W45b3 AG10 (Wheat, US)
10Rs AG5 (Soybean, Japan)
(ZG-3)R1013 AG11 (Lupine, Australia)
22Rs AGBI (Soil, Japan)
TE 2-4 AGBI (Soil, Japan)
(ZG1-2)SA50 AG8 (Oats, Australia)
(ZG1-3)SA1512 AG8(Barley, Australia)
(ZG1-5)92547 AG8 (Barley, Australia)
116Rs AG9 (Potato, US)
BN38 CAG4 (Soybean, US)
72Rs AG6 HG-I (Soil, Japan)
74Rs AG6 GV (Soil, Japan)
BN22 CAG7 (Pittosprum, US)
SIR-1 AGF (Sweetpotato, Japan)
BN37 CAG5 (Cucumber, US)
Strict consensus of
6 trees
Length = 2028
CI = 0.3743
RI = 0.5572


B
C-662 AGA (Soil, Japan)
SIR-2 AGBo (Sweetpotato, Japan)
AGG
FK02-1 AGL (Soil, Japan)
FK06-2 AGO (Soil, Japan)
STC-9 AGH (Soil, Japan)
C-610 AGD (Unknown, Japan)
BN1 CAG1 (Turfgrass, US)
C-620 AGQ (Soil, Japan)
C-460 AGBa (Rice, Japan)
C-455 AGBb (Rice, Japan)
AG7(76RS)
BN31 CAG3 (Peanut, US)
1Rs AG1-IA (Soybean, US)
36Rs AG1-IB (Turfgrass, US)
3Rs AG1-IC (Pine, Canada)
8Rs AG2-1 (Potato, US)
(ZG1-3)SA1512 AG8 (Barley, Australia)
(ZG-3)R1013 AG11 (Lupine, Australia)
(ZG9)91614 AG10 (Barley, Australia)
W45b3 AG10 (Wheat, US)
4Rs AG3 (Potato, US)
10Rs AG5 (Soybean, Japan)
116Rs AG9 (Potato, US)
BN38 CAG4 (Soybean, US)
BN74 CAG6 (Erigeron, US)
BN22 CAG7 (Pittosprum, US)
AG2-2(9RS)
AG2-2 IV (16RS)
72Rs AG6 HG-I (Soil, Japan)
74Rs AG6 GV (Soil, Japan)
(ZG1-2)SA50 AG8 (Oats, Australia)
(ZG1-5)92547 AG8- (Barley, Australia)
22Rs AGBI (Soil, Japan)
TE 2-4 AGBI (Soil,Japan)
SIR-1 AGF (Sweetpotato, Japan)
BN37 CAG5 (Cucumber, US)
18Rs AG4 HGII (Sugarbeet, Japan)
30Rs AG4 HGII (Unknown, Canada)
7Rs AG4 HGII (Alfalfa, US)
AH-1 AG4HGI (Peanut, Japan)
6Rs AG4 HGIII (Conifer, US)
45Rs AG4 HGIII (Sugarbeet, US)
Strict consensus of
177 trees
Length = 1190
CI = 0.3697
RI = 0.5495



C
C-662 AGA (Soil, Japan)
SIR-2 AGBo (Sweetpotato, Japan)
AGG
FK02-1 AGL (Soil, Japan)
FK06-2 AGO (Soil, Japan)
STC-9 AGH (Soil, Japan)
C-610 AGD (Unknown, Japan)
BN1 CAG1 (Turfgrass, US)
C-620 AGQ (Soil, Japan)
C-460 AGBa (Rice, Japan)
C-455 AGBb (Rice, Japan)
BN31 CAG3 (Peanut, US)
1Rs AG1-IA (Soybean, US)
36Rs AG1-IB (Turfgrass, US)
3Rs AG1-IC (Pine, Canada)
AG7(76RS)
(ZG1-3)SA1512 AG8 (Barley, Australia)
(ZG1-2)SA50 AG8 (Oats, Australia)
(ZG1-5)92547 AG8 (Barley, Australia)
(ZG-3)R1013 AG11 (Lupine, Australia)
(ZG9)91614 AG10 (Barley, Australia)
W45b3 AG10 (Wheat, US)
72Rs AG6 HG-I (Soil, Japan)
74Rs AG6 GV (Soil, Japan)
SIR-1 AGF (Sweetpotato, Japan)
BN37 CAG5 (Cucumber, US)
10Rs AG5 (Soybean, Japan)
BN74 CAG6 (Erigeron, US)
BN38 CAG4 (Soybean, US)
BN22 CAG7 (Pittosprum, US)
AH-1 AG4HG1 (Peanut, Japan)
6Rs AG4 HGIII (Conifer, US)
45Rs AG4 HGIII (Sugarbeet, US)
18Rs AG4 HGII (Sugarbeet, Japan)
30Rs AG4 HGII (Unknown, Canada)
7Rs AG4 HGII (Alfalfa, US)
4Rs AG3 (Potato, US)
8Rs AG2-1 (Potato, US)
116Rs AG9 (Potato, US)
AG2-2(9RS)
AG2-2 IV 16RS)
22Rs AGBI (Soil, Japan)
TE 2-4 AGBI (Soil,Japan)
Strict consensus of
24 trees
Length = 1674
CI = 0.4337
RI = 0.5734



D
C-662 AGA (Soil, Japan)
SIR-2 AGBo (Sweetpotato, Japan)
AGG
FK02-1 AGL (Soil, Japan)
FK06-2 AGO (Soil, Japan)
STC-9 AGH (Soil, Japan)
C-610 AGD (Unknown, Japan)
BN1 CAG1 (Turfgrass, US)
C-620 AGQ (Soil, Japan)
C-460 AGBa (Rice, Japan)
C-455 AGBb (Rice, Japan)
AG7(76RS)
BN31 CAG3 (Peanut, US)
1Rs AG1-IA (Soybean, US)
36Rs AG1-IB (Turfgrass, US)
3Rs AG1-IC (Pine, Canada)
(ZG1-3)SA1512 AG8 (Barley, Australia)
(ZG1-2)SA50 AG8 (Oats, Australia)
(ZG1-5)92547 AG8 (Barley, Australia)
(ZG-3)R1013 AG11 (Lupine, Australia)
(ZG9)91614 AG10 (Barley, Australia)
W45b3 AG10 (Wheat, US)
72Rs AG6 HG-I (Soil, Japan)
74Rs AG6 GV (Soil, Japan)
SIR-1 AGF (Sweetpotato, Japan)
BN37 CAG5 (Cucumber, US)
10Rs AG5 (Soybean, Japan)
BN74 CAG6 (Erigeron, US)
BN38 CAG4 (Soybean, US)
BN22 CAG7 (Pittosprum, US)
AH-1 AG4HG1 (Peanut, Japan)
6Rs AG4 HGIII (Conifer, US)
45Rs AG4 HGIII (Sugarbeet, US)
18Rs AG4 HGII (Sugarbeet, Japan)
30Rs AG4 HGII (Unknown, Canada)
7Rs AG4 HGII (Alfalfa, US)
8Rs AG2-1 (Potato, US)
116Rs AG9 (Potato, US)
4Rs AG3 (Potato, US)
AG2-2(9RS)
AG2-2 IV (16RS)
22Rs AGBI (Soil, Japan)
TE 2-4 AGBI (Soil,Japan)
Strict consensus of
15 trees
Length = 1590
CI = 0.3975
RI = 0.5653

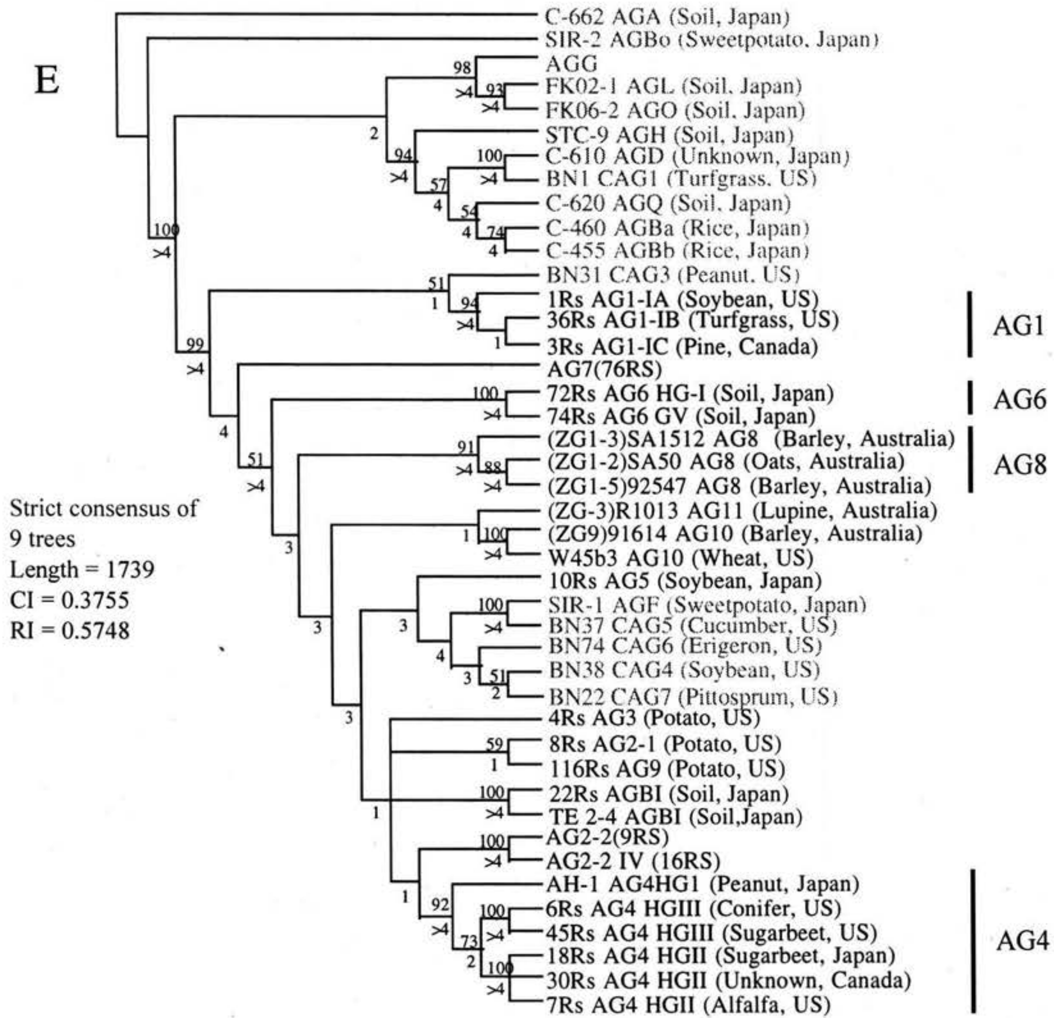


FIG 2. Strict consensus trees resulting from including indels in the analyses of three gene partitions. A) included as ambiguous characters ("?"); B) excluded; C) recoded as multistate; D) recoded as multistate to identify a specific length of the indel; E) recoded as different characters for each distinct sequence. In black, *Rhizoctonia solani* isolates (teleomorph=*Thanatephorus*), in gray binucleate *Rhizoctonia* (teleomorph=*Ceratobasidium*).

DISCUSION GENERAL

Clasificación de *Rhizoctonia solani.*

El concepto filogenético de especie puede ser una alternativa para delimitar especies en los hongos anamorfos como en el caso de *Rhizoctonia solani*. Este concepto no se apoya en un sólo criterio como interesterilidad o morfología sino que es pluralista. Primero se reconocen las especies mediante el análisis cladístico y posteriormente se toma en consideración un componente de agrupamiento (monofilia) y un componente de rango (categorización) para delimitarlas; el cual se puede decidir bajo algún criterio biológico como aislamiento reproductivo, biogeográfico o ecológico o alguna distancia morfométrica.

Los hongos anamorfos se han excluido de la aplicación de la mayoría de los conceptos de especie y consecuentemente, de los estudios de especiación a pesar de que los linajes que se reproducen asexualmente son comunes no sólo en hongos, sino en muchos grupos de organismos. Por este motivo, existe la necesidad de reconsiderar: 1) qué importancia tiene la información de los estados sexuales y 2) la base teórica de los conceptos no filogenéticos de especie. Mishler y Budd (1990) sugirieron que el estudio de especiación asexual puede ayudar a entender los mecanismos evolutivos en las especies sexuales. Los hongos anamorfos, por lo regular muy diversos, pueden ser un sistema modelo para explorar estos dos puntos.

La existencia de hongos anamorfos es un problema serio en la sistemática de los hongos en general. Sin embargo, desde hace algunos años, los micólogos interesados en su clasificación han usado caracteres de los ácidos nucleicos. Aunque han tenido éxito relativo (Taylor, 1995), todavía se necesitan analizar muchas especies más, a la luz de la sistemática filogenética para ubicar a los miembros de estos hongos en su correspondiente grupo taxonómico. El hongo anamorfo *Rhizoctonia solani* ha existido como taxón por más de cien años. No hay duda que erigir nuevas especies o proponer rearreglos dentro de la taxonomía de este hongo va a resultar en resistencia por los usuarios, sean científicos o no, ya que toda la literatura asociada a éste, como claves y otras publicaciones resultaría obsoleta para identificarlo. Sin embargo, a largo plazo, una clasificación basada en las relaciones filogenéticas resultaría en una clasificación mas robusta y estable para este importante hongo fitopatógeno.

Las secuencias de las regiones intergénicas (ITS1 y 2), la subunidad larga (LSU) del ADN ribosomal y el gen beta-tubulina, analizadas bajo un enfoque cladista, nos permitieron delimitar solo parcialmente el número de especies dentro del complejo de *R. solani*. Nuestros resultados mostraron ramas contradictorias o poco apoyadas en algunos de los análisis realizados. Sin embargo, el apoyo a la mayoría de los clados aumentó cuando se combinaron los tres juegos de datos. Además, se demostró que parte de la información histórica se pierde cuando se excluyen las indels de los análisis. Por otro lado, siempre se recobraron dos grandes clados, uno que contiene solo grupos anastomósicos de *Ceratobasidium* el cual es el grupo hermano de todos los grupos anastomósicos de *R. solani* (teleomorph = *Thanatephorus*) y seis de *Ceratobasidium*, lo que mantiene la hipótesis de que *Thanatephorus* es parafilético con respecto a *Ceratobasidium*. Esto sugiere que ni el número de núcleos por célula ni la reacción de anastomosis son caracteres diagnósticos de estos géneros como previamente se había juzgado.

El hecho de que los clados no se mantuvieron estables en la mayoría de los análisis reveló que es necesario hacer un muestreo más amplio, sobre todo con aislamientos de *Ceratobasidium*, e incluir otro marcador molecular que refuerce el apoyo en las ramas

basales para obtener una hipótesis filogenética más robusta y para definir con mayor claridad cuantas especies hay en estos dos grupos. Por ahora, no es posible refutar la hipótesis de que cada grupo anastomósico representa una especie. Sin embargo, he tomado una decisión conservadora y recomiendo que se reconozcan como especies diferentes solo los cuatro clados formados por aislamientos de los grupos anastomósicos 1, 4, 6 y 8 de *R. solani* (teleomorfo = *Thanatephorus*) respectivamente (Fig. 2, página 57). Los nombres científicos disponibles serían *Thanatephorus sasaki* (Shirai) Tu & Kimbrough para el clado que incorpora los aislamientos del grupo anastomósico 1 (AG1) y *T. praticola* (Kotila) Flentje para el clado que incluye los aislamientos del grupo anastomósico 4 (AG4). Para el clado que incluye los aislamientos del grupo anastomósico 6 (AG6) así como el clado formado con aislamientos del grupo anastomósico 8 (AG8) no existe nombre científico disponible (en preparación).

Distinción filogenética entre *Thanatephorus* y *Ceratobasidium*.

En la actualidad todavía hay desacuerdo en la taxonomía de *Thanatephorus* y *Ceratobasidium* y su ubicación filogenética inclusive dentro de los Basidiomycota. Por el momento, los resultados de este estudio señalaron que algunos aislamientos de *Ceratobasidium* están más relacionados con aislamientos de *Thanatephorus* que con otros del mismo género.

Las características vegetativas de los distintos teleomorfos de *Rhizoctonia* como el color del micelio, el diámetro de la hifa, el número de núcleos, la longitud de las células, la forma y tamaño de las células monilioides y el tamaño de los esclerocios (Apéndice I) son ampliamente utilizadas para definir especies. Sin embargo, estas características varían considerablemente con cambios en la temperatura, luz y la composición del medio de cultivo. De hecho, se ha observado que muchos aislamientos de *Thanatephorus* y *Ceratobasidium* a menudo tienen apariencia similar en el medio de cultivo (Andersen, 1990; Stalpers y Andersen, 1996), y que en algunos casos algunos aislamientos de estos dos géneros presentan fusión de hifas y número variable de núcleos por célula (Kotila, 1929; Parmeter *et al.*, 1967; Burpee *et al.*, 1980; Ogoshi, 1987). Esto es evidencia de que ciertos aislamientos de *Ceratobasidium* y *Thanatephorus* están estrechamente relacionados.

Existen muy pocos estudios taxonómicos en donde hayan incluido aislamientos de *Ceratobasidium* y *Thanatephorus*. Johanson *et al.* (1998) usando secuencias del ITS encontraron que los aislamientos de *Ceratobasidium oryzae-sativae* (anamorfo = *Rhizoctonia oryzae-sativae*, grupo anastomósico AGBb) están más relacionados con *T. cucumeris* (anamorfo = *Rhizoctonia solani*, grupo anastomósico AG1) que con *Waitea circinata* (anamorfo = *Rhizoctonia oryzae*, grupo anastomósico WAG-O). También Boidin *et al.*, (1998) usando secuencias del ITS concluyeron que *Ceratobasidium* está cercanamente relacionado a *Thanatephorus*.

En este trabajo de tesis se extendieron los estudios previos al incluir secuencias no solo de la región del ITS del ADN ribosomal nuclear, sino además una sección de la subunidad larga (LSU) y una porción del gen del beta-tubulina. También se incluyeron un mayor número de aislamientos de los distintos grupos anastomósicos tanto binucleados (*Ceratobasidium*) como multinucleados (*Thanatephorus*). Los resultados derivados de este análisis sugirieron que algunos aislamientos actualmente clasificados en *Ceratobasidium* con base en el número de núcleos y la anastomosis de hifas, deben estar clasificados dentro de *Thanatephorus*. Sin embargo, es necesario incluir, en un análisis posterior, más

aislamientos de *Ceratobasidium* especialmente de los grupos anastomósicos CAG3, CAG4, CAG5, CAG6, CAG7, y AGF para substanciar la relación de éstos con *Thanatephorus*.

Valor filogenético de las indels

En cualquier clase de datos, los caracteres son las unidades a través de las cuales comunicamos las idea de homología, relación, diagnosis e identidad (Forey y Kitching, 2000). En el caso de secuencias génicas de longitud desigual, cualquier error en el alineamiento podría conducirnos a hipótesis de homología erróneas y consecuentemente a conclusiones taxonómicas equivocadas.

La base teórica para el análisis cladístico de las secuencias génicas al igual que para cualquier otro tipo de caracteres es el concepto de homología filogenética. Bajo este enfoque conceptual en el caso de las secuencias, si los nucleótidos en una posición no varían, éstos son evidencia de homología táxica y si las bases son diferentes, entonces se infiere una hipótesis de homología transformacional. Este principio conceptual se aplica tanto a las substituciones como a las indels que se generan durante el alineamiento de secuencias de diferente longitud. Tal como en el caso de los caracteres morfológicos, la congruencia entre hipótesis resolverá si la similitud observada en las bases o en las indels en una posición de la secuencia puede mantenerse como evidencia de homología filogenética.

La elaboración de hipótesis de homología en el ámbito de secuencias génicas de longitud desigual es un proceso complejo que incorpora las bases empíricas de similitud, variación discreta, conjunción, heredabilidad e independencia. También, incluye una fase inferencial en la que las homologías potenciales se evalúan si son congruentes en un cladograma más parsimonioso. Todo esto hace que el análisis de las secuencias génicas requiera no solo una base empírica amplia, sino también un marco teórico claro.

Las filogenias moleculares recaen predominantemente o exclusivamente en substituciones. Sin embargo, desde el punto de vista molecular, las inserciones-deleciones (indels) también se originan de eventos biológicos particulares como mutaciones, de tal manera que pueden contener la misma información histórica que observamos en las substituciones (Giribert y Wheeler, 1999). De hecho, ya se ha sugerido que las indels de varios nucleótidos contiguos son valiosas para identificar grupos monofilético (Lloyd y Calder, 1991, Kawakita *et al*., 2003).

En algunos estudios donde las indels se han considerado como mutaciones, se incorporan en los análisis como una clase particular de caracteres filogenéticos (Lutzoni *et al*., 2000; Simmons y Ochoterena, 2000). Sin embargo, éstas no han sido consideradas ampliamente como marcadores filogenéticos. Esto se debe a que en la actualidad no hay suficientes estudios empíricos en donde se evalúe la calidad de las indels como una fuente de información filogenética (Kawakita *et al*., 2003). Sin duda, y al igual que con caracteres morfológicos complejos, pueden haber múltiples formas para codificar las indels. La estrategia precisa todavía no se ha establecido, pero incluirlas en los análisis nos da la oportunidad de evaluarlas como hipótesis que están sujetas a la prueba de congruencia en un análisis de parsimonia (Apéndice II). En este estudio precisamente se demostró que parte de la información histórica se pierde cuando se excluyen las indels de los análisis. Por lo tanto, es importante que los estudios con secuencias de longitud desigual incorporen una evaluación del efecto de incluir las indels en la estabilidad de las hipótesis filogenéticas, debido a que pueden ser buenos caracteres filogenéticos, especialmente a los niveles

taxonómicos menos inclusivos; pero también reconozco que, al igual que otras clases de caracteres, pueden incorporar homoplasias a los análisis.

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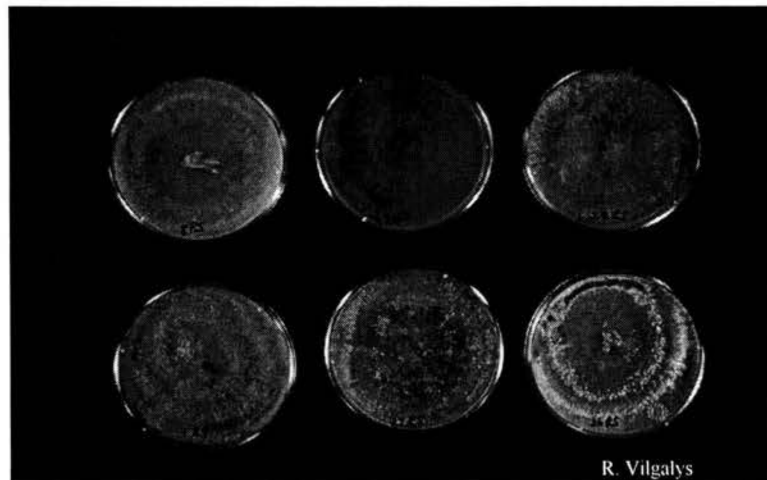
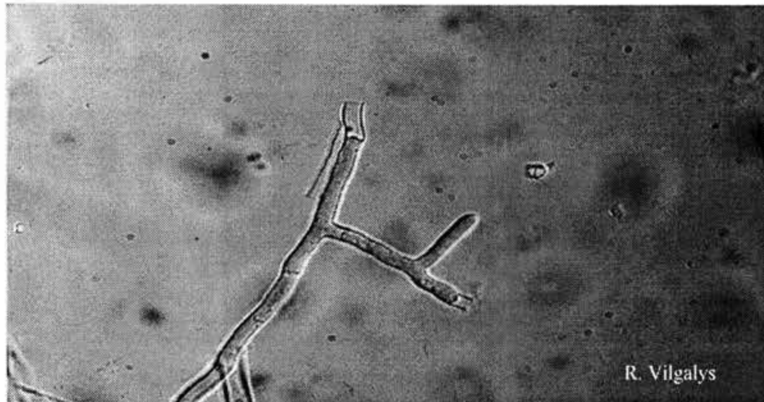
Apéndice I. Caracteres morfológicos usados para identificar aislamientos de *R. solani*

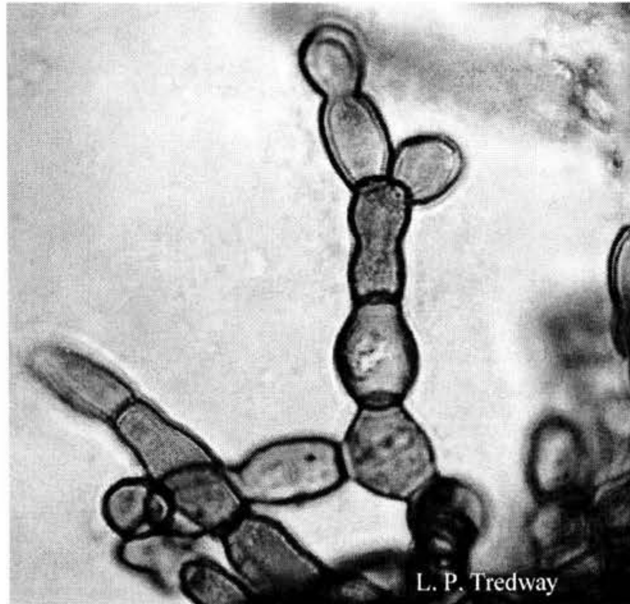
Micelio amarillo pálido a café oscuro de crecimiento rápido

Esclerocios de tamaño y forma variable

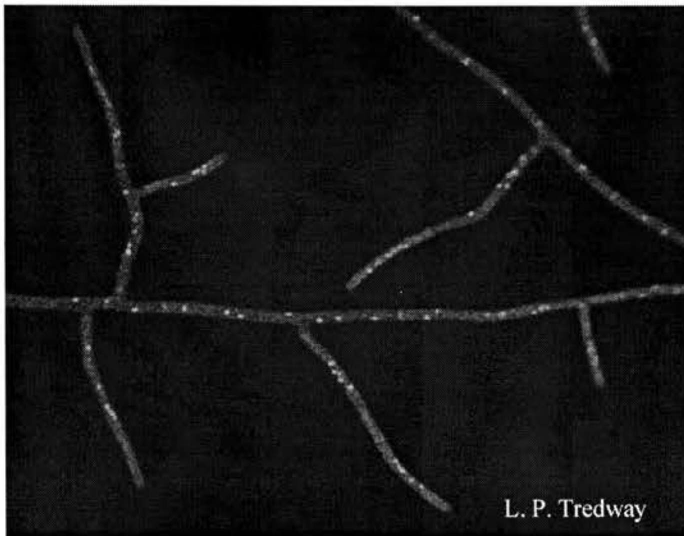




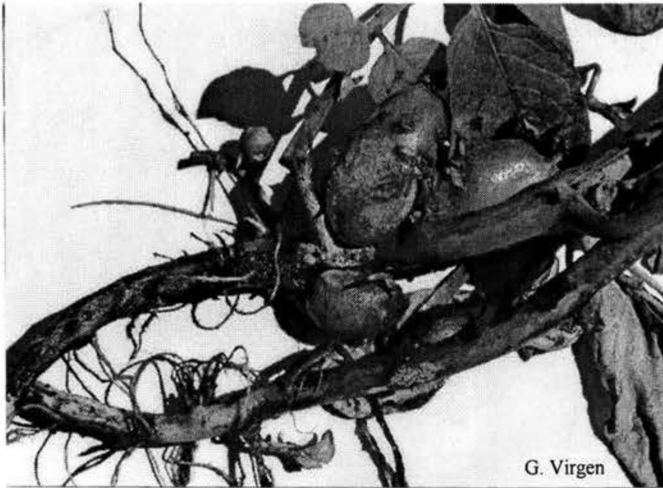
Hifas de más de 7 µm de diámetro, estrechas en los septos, a menudo ramificadas en ángulo recto



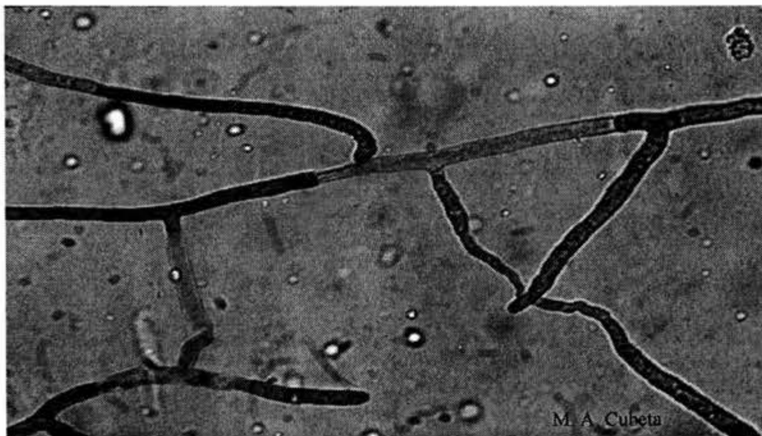
Células monilioides



Células multinucleadas

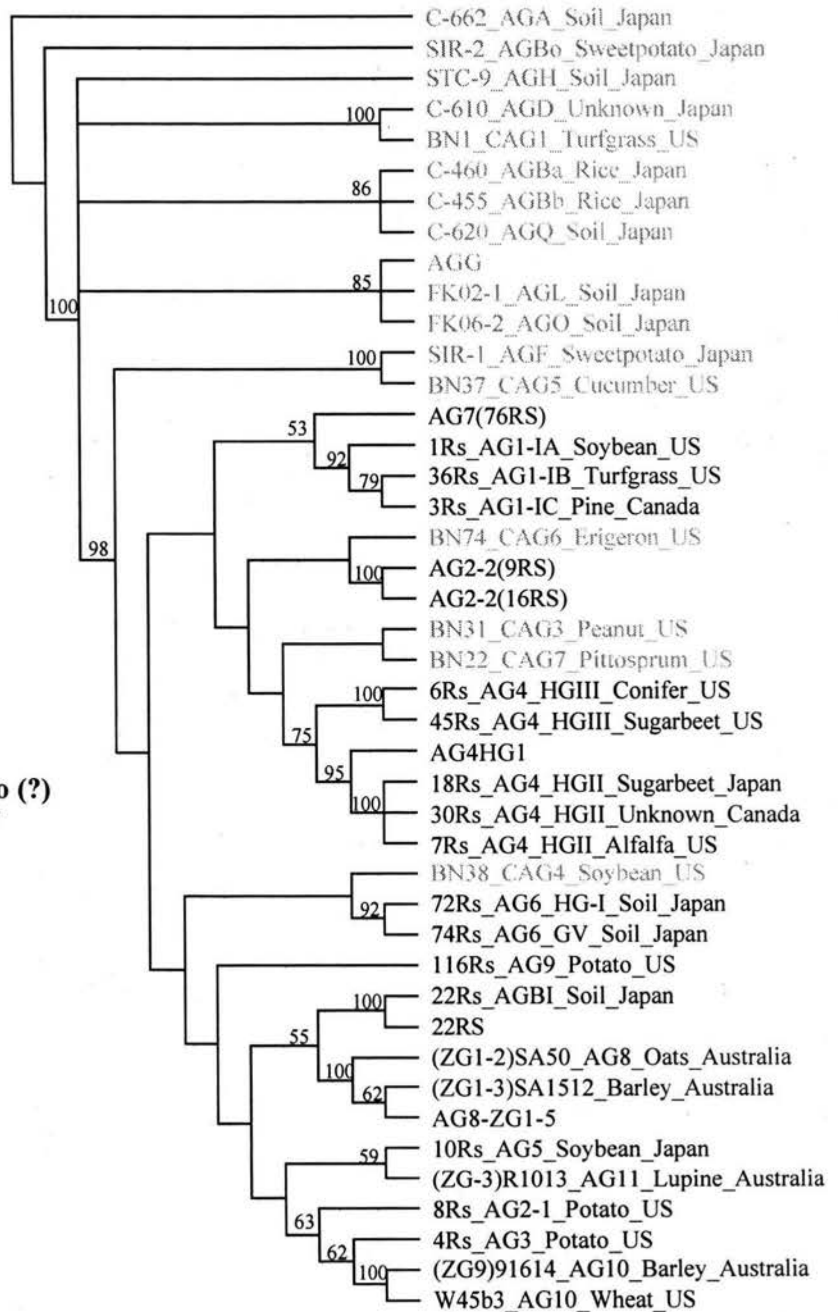


Patógeno, Simbionte
o Saprófito

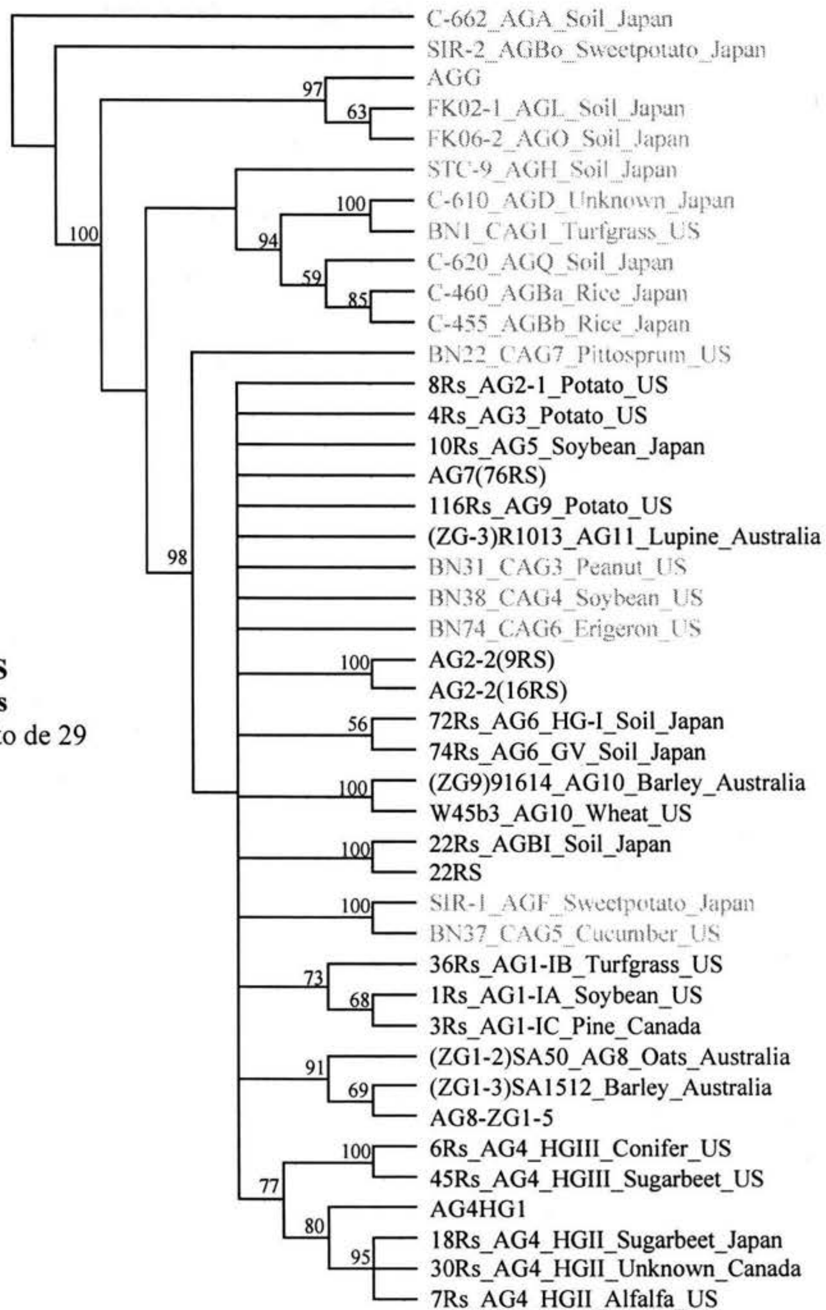


Anastomosis de hifas

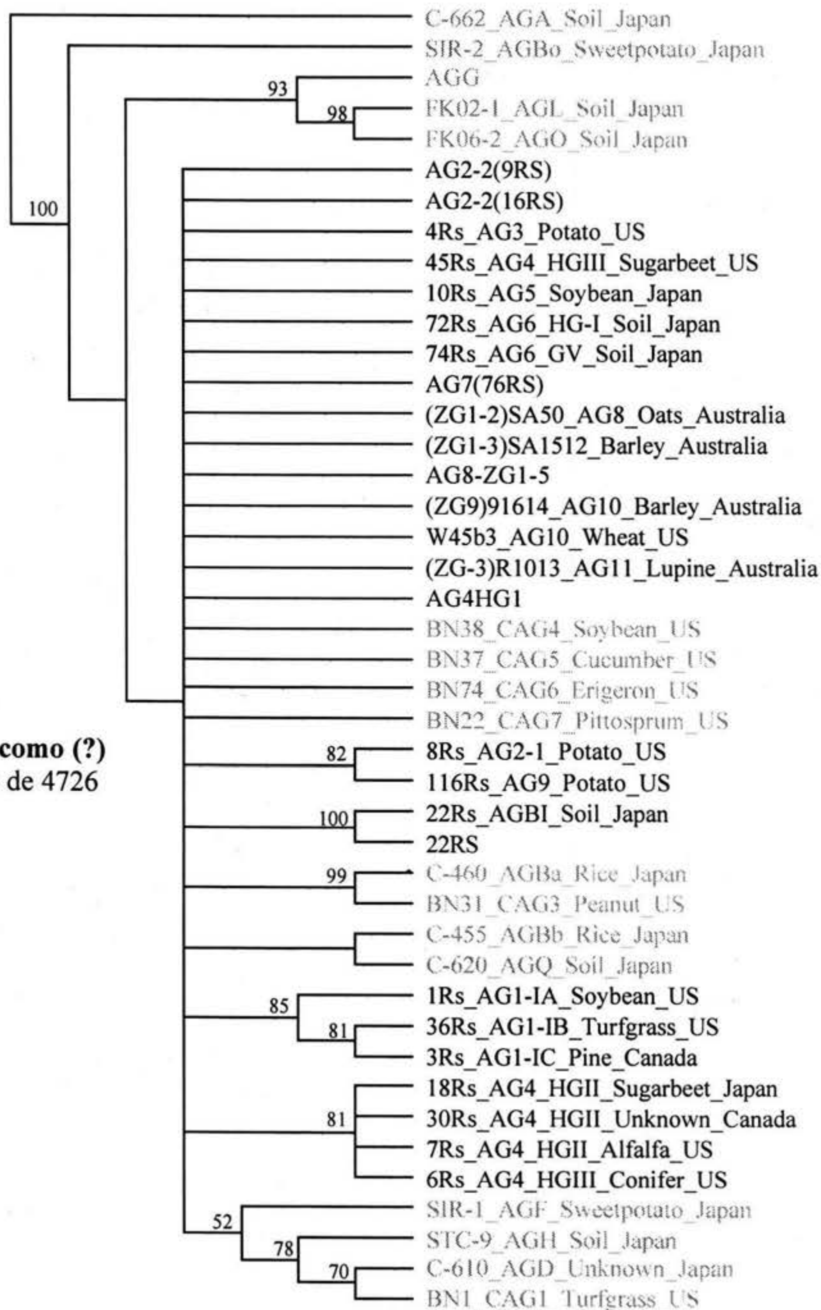
Apéndice II. Cladogramas obtenidos con el análisis de cada juego de datos y en combinación, cuando las indels son incluidas como caracteres ambiguos (?) y cuando son excluidas



Análisis del ITS
Indels incluidas como (?)
Consenso estricto de 7 árboles
CI: 0.4191
RI: 0.6240

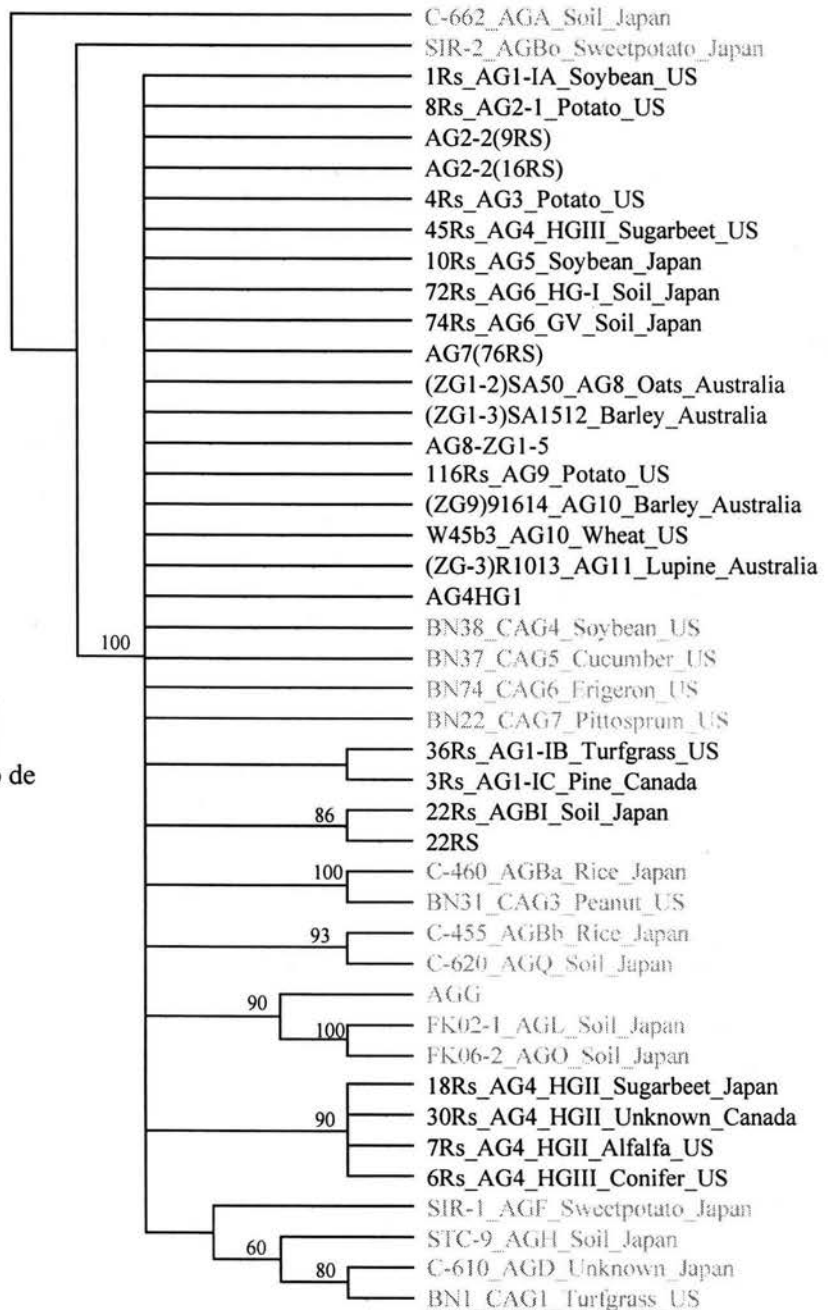


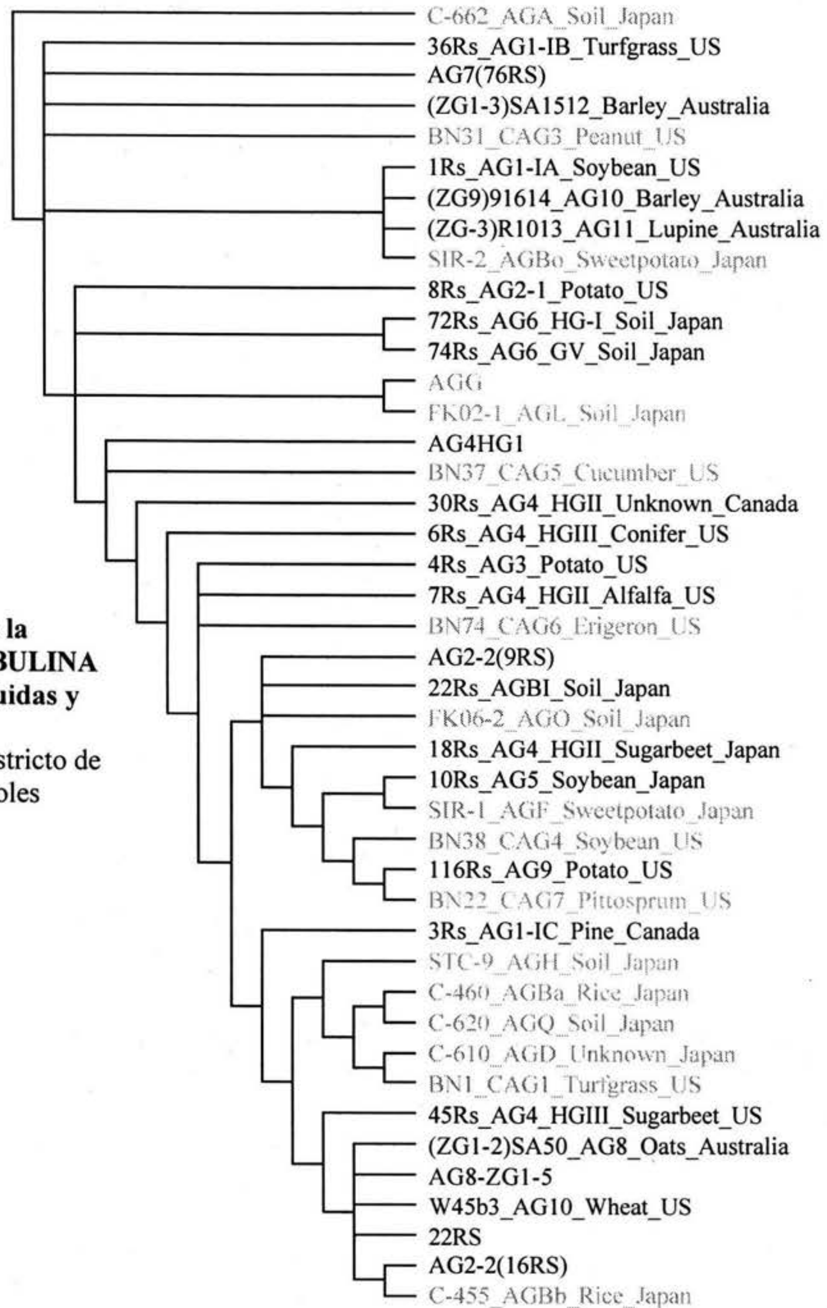
Análisis del ITS
Indels excluidas
Consenso estricto de 29 árboles
CI: 0.4603
RI: 0.6764



Análisis de LSU
Indels incluidas como (?)
Consenso estricto de 4726 árboles
CI: 0.4737
RI: 0.6585

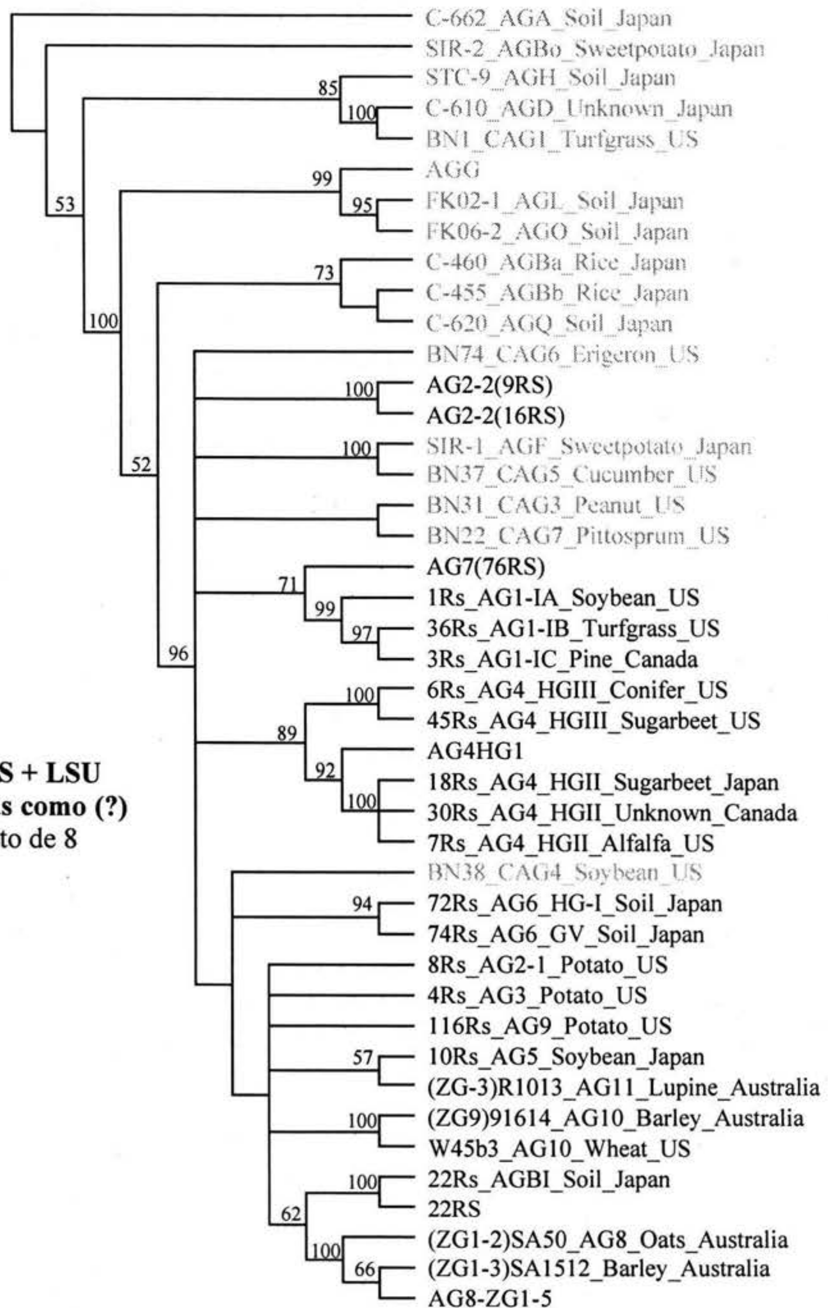
Análisis de LSU
Indels excluidas
Consenso estricto de
11323 árboles
CI: 0.4842
RI: 0.6535

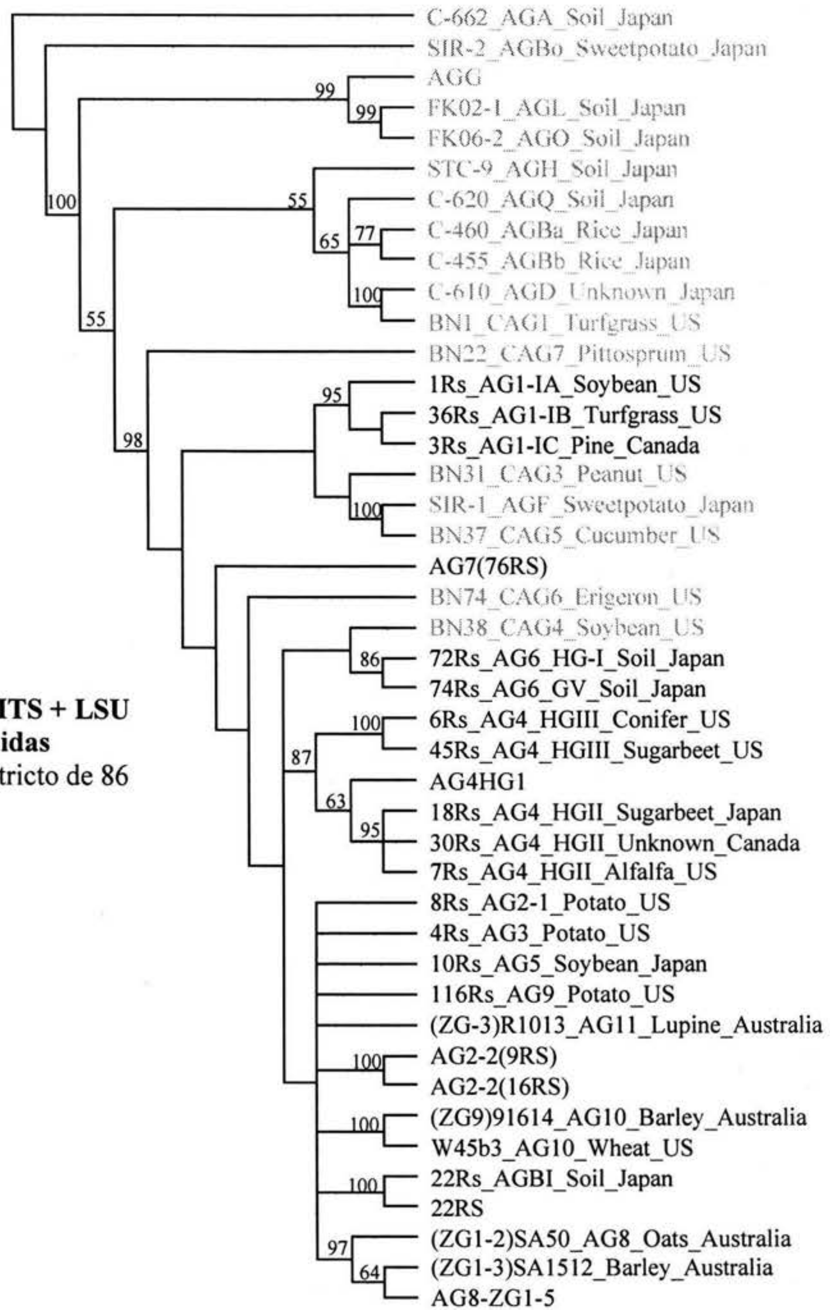




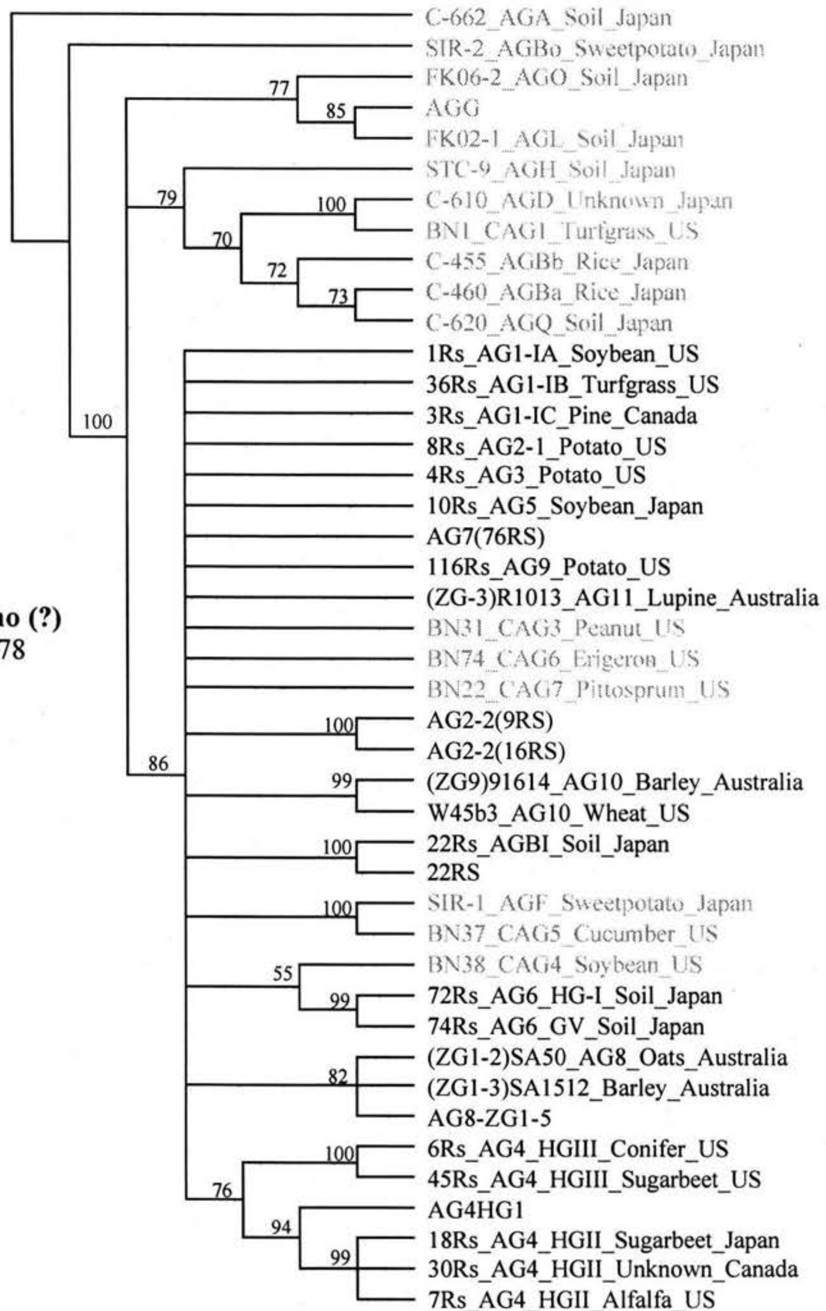
Análisis de la BETA-TUBULINA Indels incluidas y excluidas
Consenso estricto de 113776 árboles
CI: 0.4084
RI: 0.7002

Análisis del ITS + LSU
Indels incluidas como (?)
Consenso estricto de 8 árboles
CI: 0.4158
RI: 0.6100

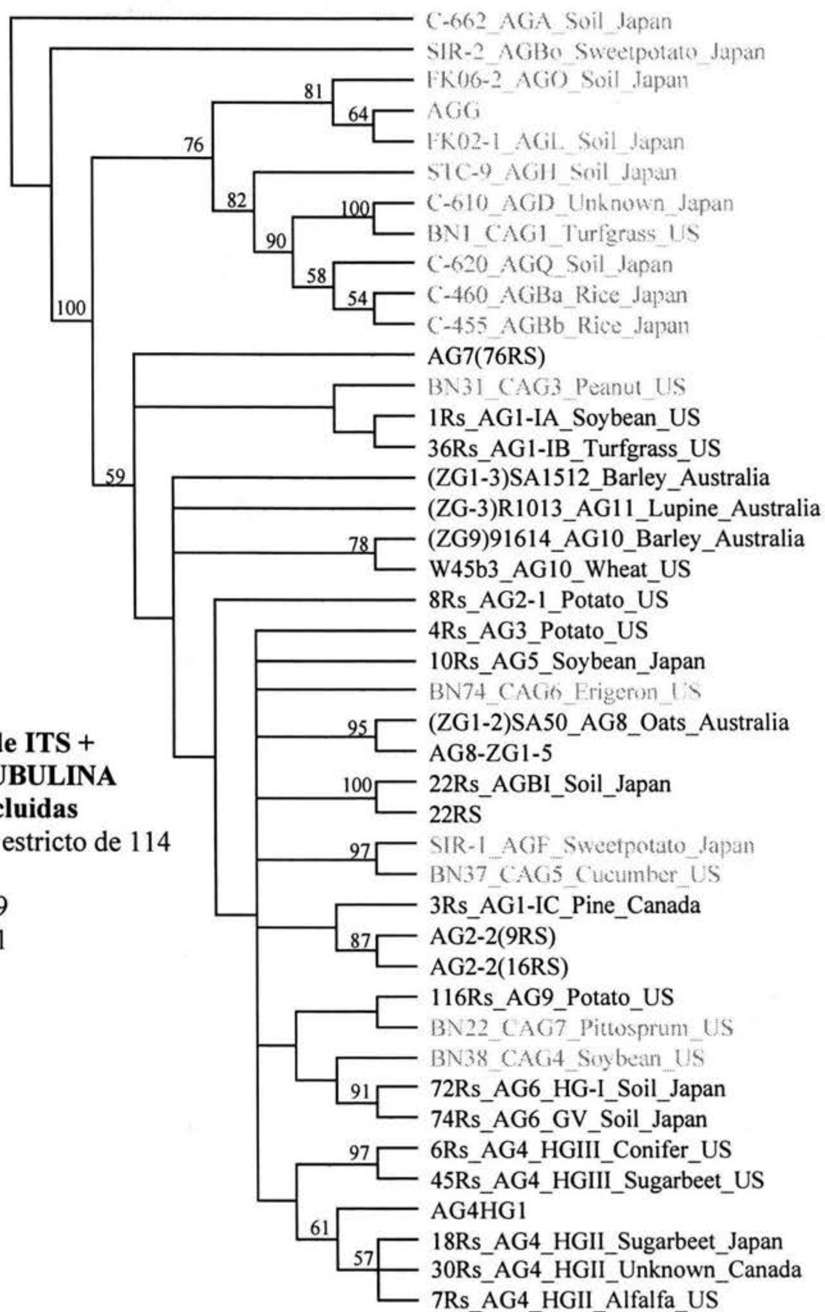




Análisis de ITS + LSU
Indels excluidas
Consenso estricto de 86 árboles
CI: 0.4416
RI: 0.6333



Análisis del ITS + BETA-TUBULINA
Indels incluidas como (?)
Consenso estricto de 78 árboles
CI: 0.3710
RI: 0.5631

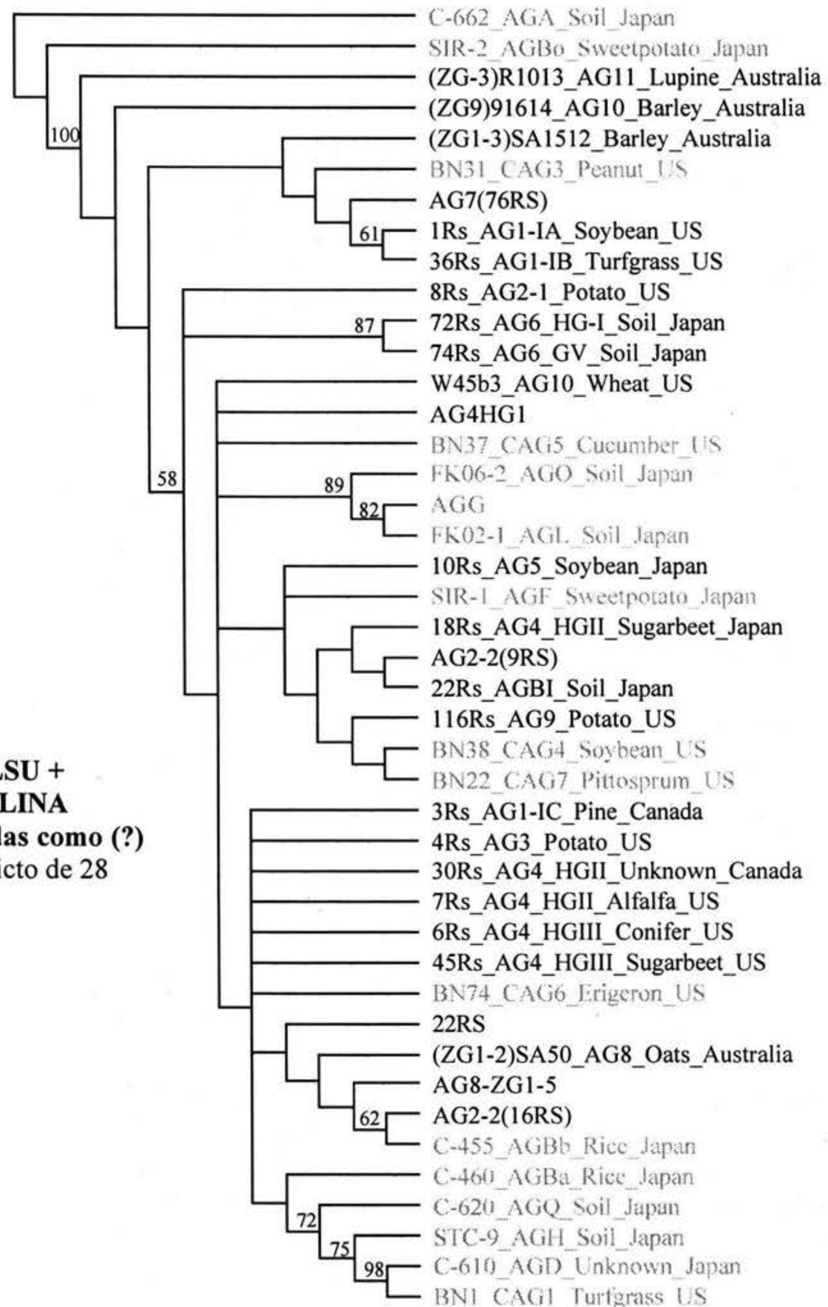


Análisis de ITS + BETA-TUBULINA Indels excluidas

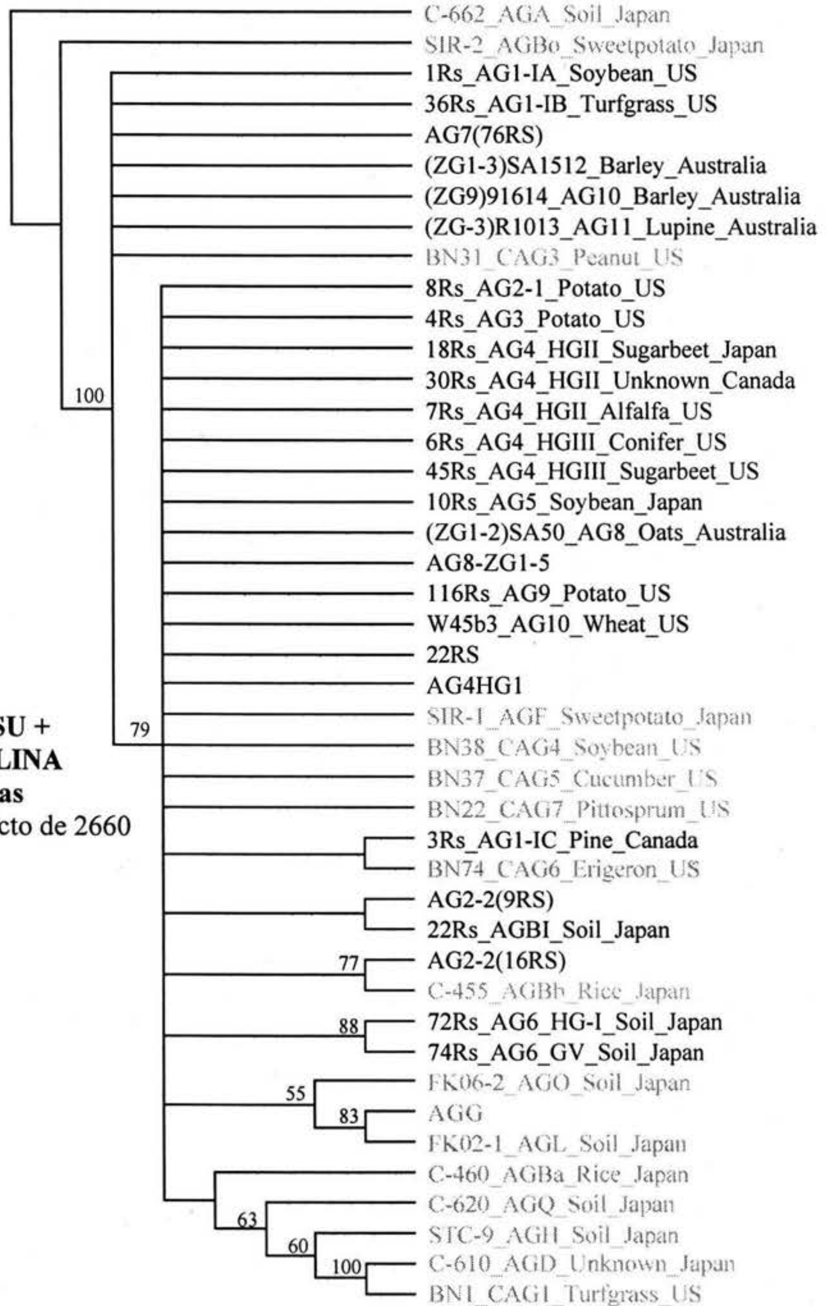
Consenso estricto de 114 árboles

CI: 0.3659

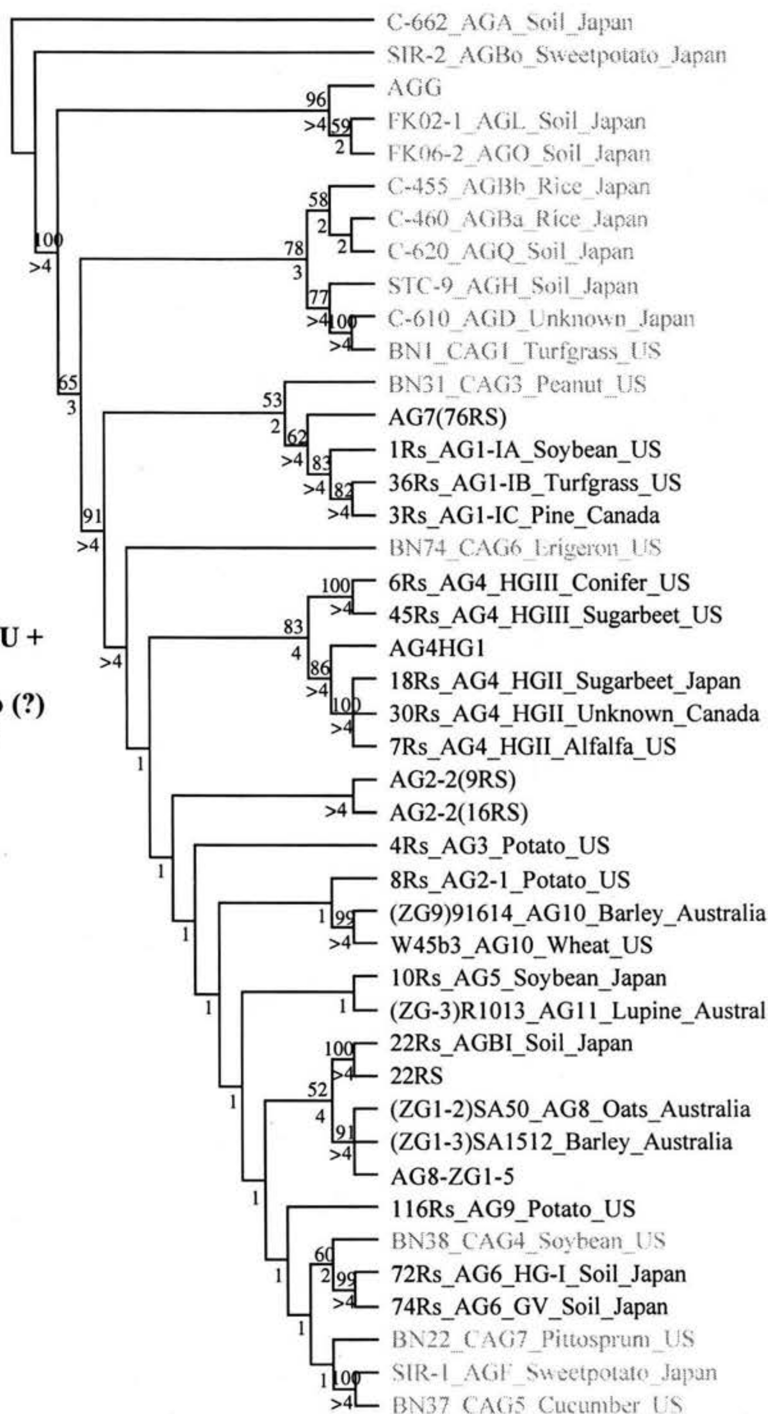
RI: 0.5681



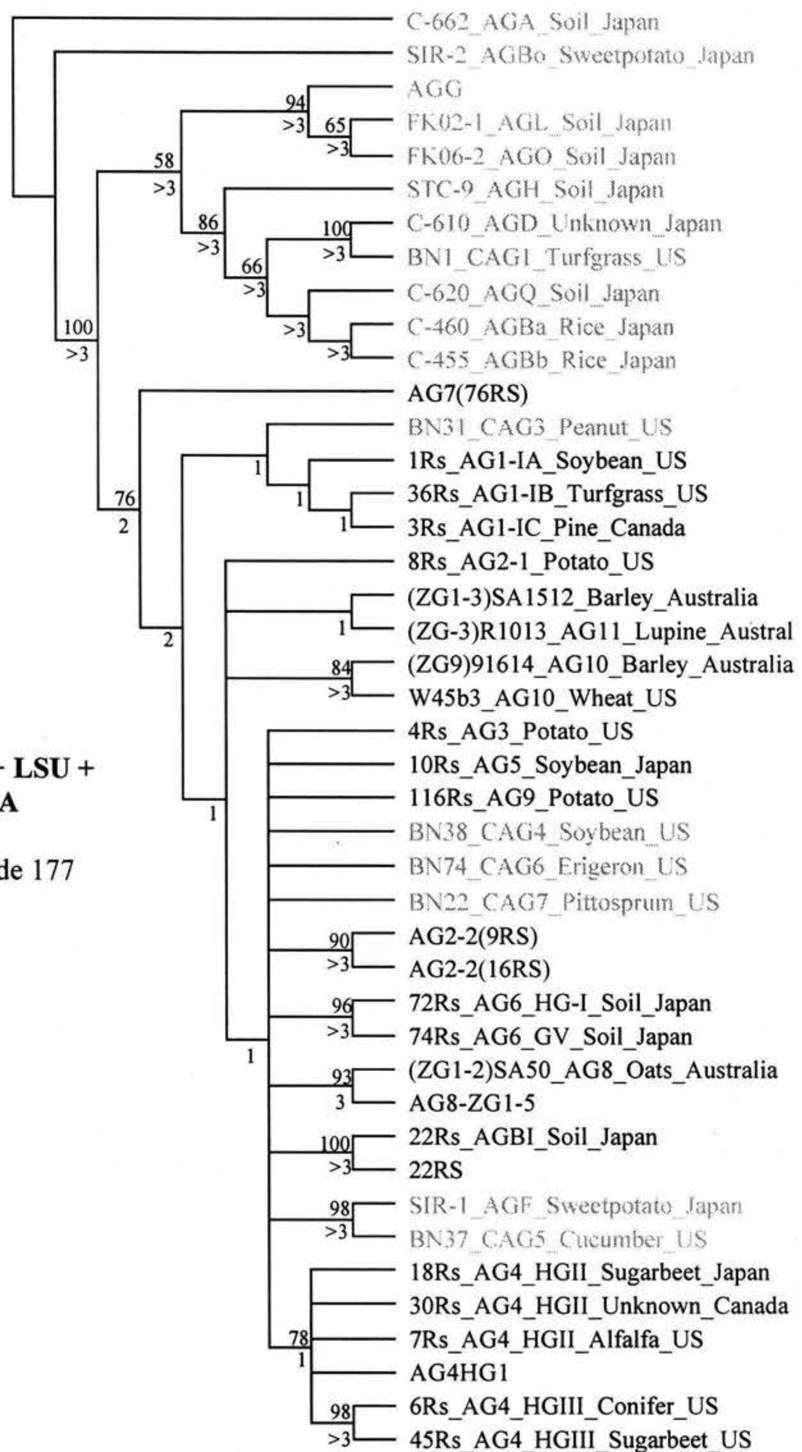
Análisis del LSU + BETA-TUBULINA Indels incluidas como (?)
Consenso estricto de 28 árboles
CI: 0.3635
RI: 0.5599



Análisis de LSU + BETA-TUBULINA
Indels excluidas
Consenso estricto de 2660 árboles
CI: 0.3690
RI: 0.5674



Análisis del ITS + LSU + BETA-TUBULINA
Indels incluidas como (?)
Consenso estricto de 6 árboles
CI: 0.3743
RI: 0.5572



Análisis del ITS + LSU + BETA-TUBULINA
Indels excluidas
Consenso estricto de 177 árboles
CI: 0.3697
RI: 0.5495

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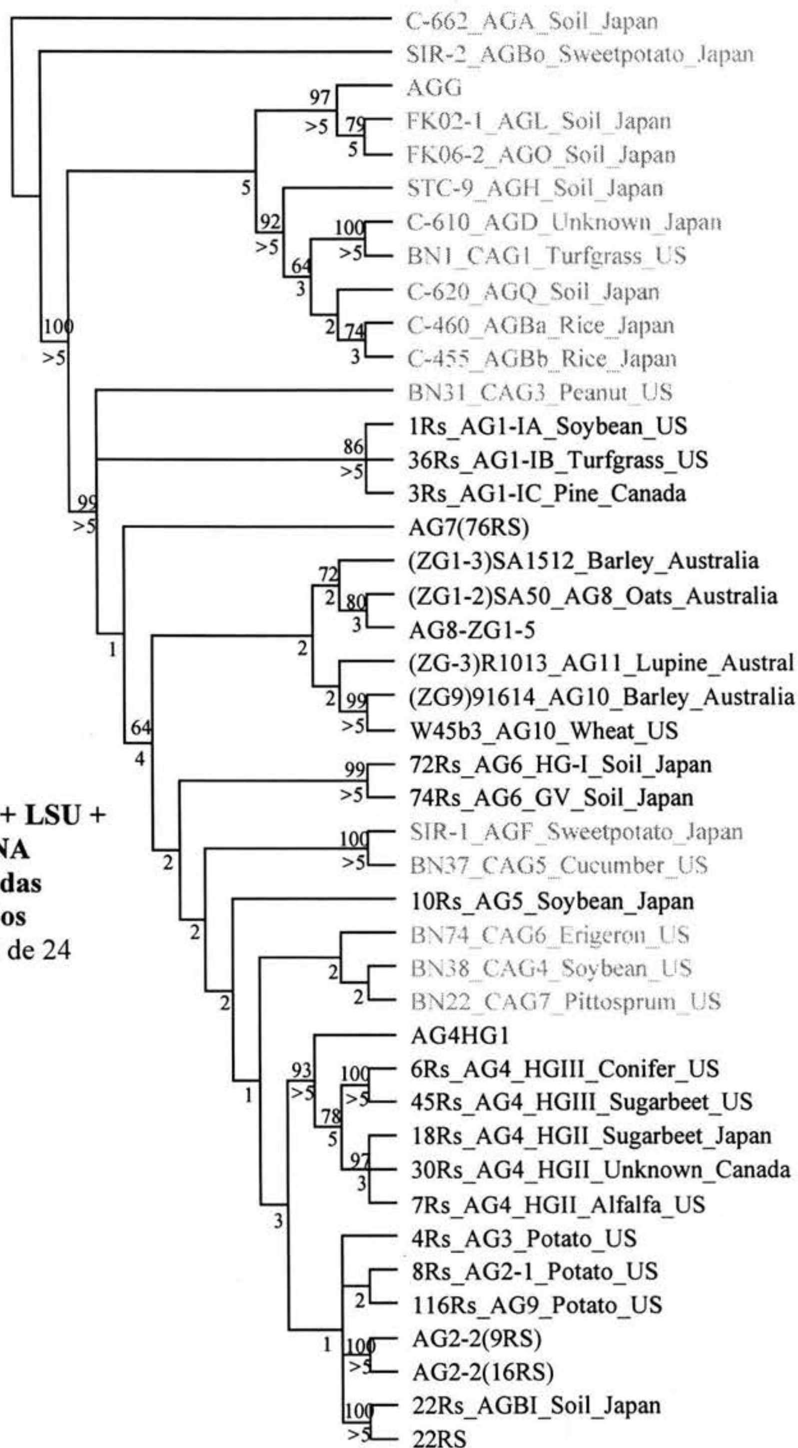
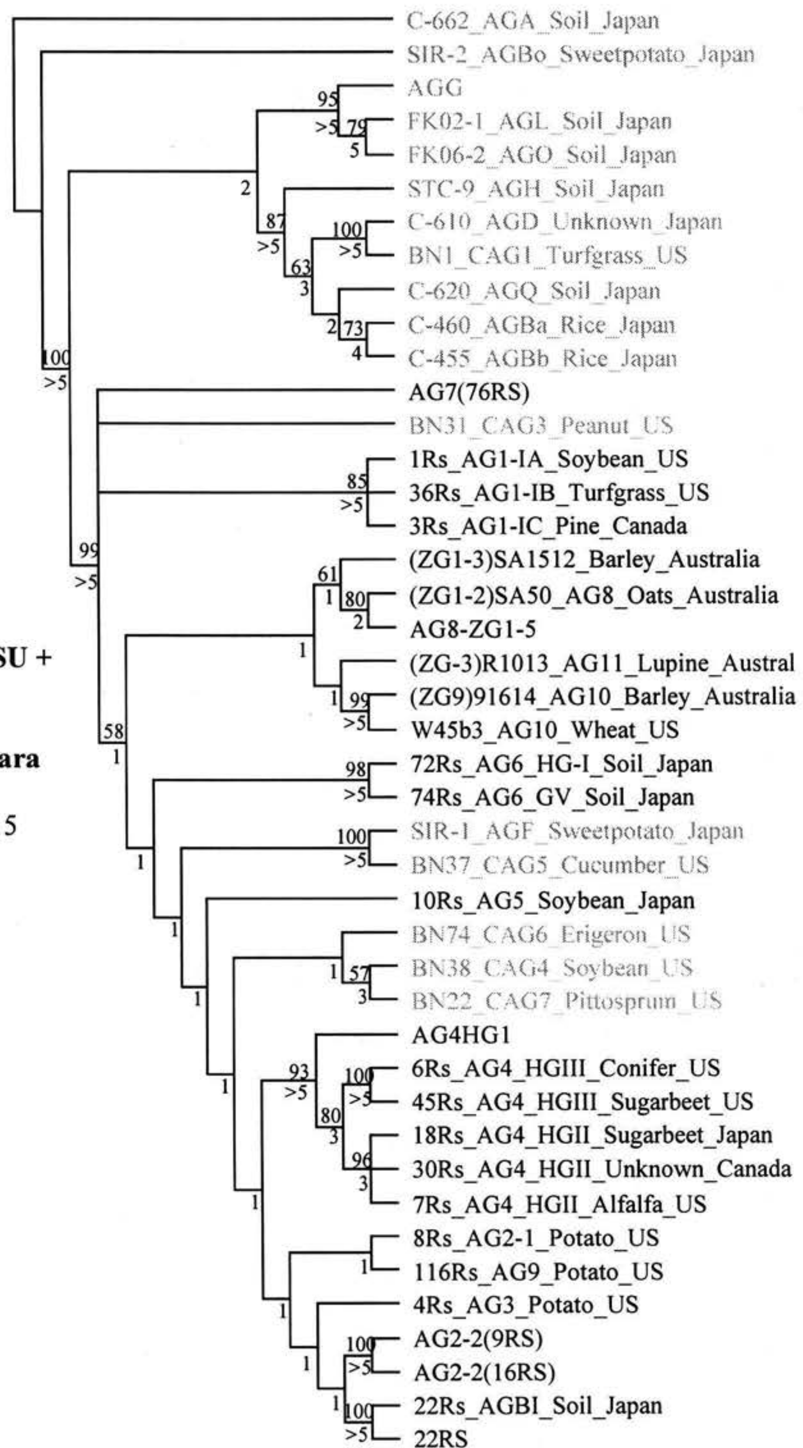
DE LA BIBLIOTECA



Análisis del ITS + LSU + BETA-TUBULINA
Indels recodificadas como multiestados
Consenso estricto de 24 árboles
CI: 0.4337
RI: 0.5734



Análisis del ITS + LSU + BETA-TUBULINA Indels recodificadas como multiestados para cada longitud
Consenso estricto de 15 árboles
CI: 0.3975
RI: 0.5653

C-662_AGA_Soil_Japan
SIR-2_AGBo_Sweetpotato_Japan
AGG
FK02-1_AGL_Soil_Japan
FK06-2_AGO_Soil_Japan
STC-9_AGH_Soil_Japan
C-610_AGD_Unknown_Japan
BN1_CAG1_Turfgrass_US
C-620_AGQ_Soil_Japan
C-460_AGBa_Rice_Japan
C-455_AGBb_Rice_Japan
BN31_CAG3_Peanut_US
1Rs_AG1-IA_Soybean_US
36Rs_AG1-IB_Turfgrass_US
3Rs_AG1-IC_Pine_Canada
AG7(76RS)
72Rs_AG6_HG-I_Soil_Japan
74Rs_AG6_GV_Soil_Japan
(ZG1-3)SA1512_Barley_Australia
(ZG1-2)SA50_AG8_Oats_Australia
AG8-ZG1-5
(ZG-3)R1013_AG11_Lupine_Australia
(ZG9)91614_AG10_Barley_Australia
W45b3_AG10_Wheat_US
10Rs_AG5_Soybean_Japan
SIR-1_AGF_Sweetpotato_Japan
BN37_CAG5_Cucumber_US
BN74_CAG6_Erigeron_US
BN38_CAG4_Soybean_US
BN22_CAG7_Pittosprum_US
4Rs_AG3_Potato_US
8Rs_AG2-1_Potato_US
116Rs_AG9_Potato_US
22Rs_AGBI_Soil_Japan
22RS
AG2-2(9RS)
AG2-2(16RS)
AG4HG1
6Rs_AG4_HGIII_Conifer_US
45Rs_AG4_HGIII_Sugarbeet_US
18Rs_AG4_HGII_Sugarbeet_Japan
30Rs_AG4_HGII_Unknown_Canada
7Rs_AG4_HGII_Alfalfa_US

Análisis del ITS + LSU + BETA-TUBULINA Indels recodificadas como diferentes caracteres
Consenso estricto de 9 árboles
CI: 0.3755
RI: 0.5748